Cellular Influx and Cytotoxicity of Oxaliplatin Analogues

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Das Gleiche läßt uns in Ruhe,

aber der Widerspruch ist es,

der uns produktiv macht.

Johann Wolfgang von Goethe

Abbreviations

ANOVA	Analysis of variance
ATP7A	Adenosine triphosphate alpha-polypeptide
ATP7B	Adenosine triphosphate beta-polypeptide
A2780	Ovarian carcinoma cell line
A2780cis	Cisplatin-resistant ovarian carcinoma cell line
BCA	Bicinchoninic acid
BSA	Bovine serum albumine
Caspase	Cysteinyl aspartate-specific proteinase
cDDP	cis-Platinum(II)-diammine-dichloride
cDNA	Complementary DNA
CTR1	Copper Transporter 1
DACH	1,2-Diaminocyclohexane
dAMP	2'-Deoxyadenosine 5'-monophosphate sodium salt
DAPI	6-Diamidino-2-phenylindole dihydrochloride
DEPC	Dietyhl pyrocarbonate
dGMP	2'-Deoxyguanosine 5'-monophosphate sodium salt
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Drug concentration that produces 50 % of the maximum possible response
EDTA	2,2',2'',2'''-(Ethane-1,2-diyldinitrilo) tetraacetic acid disodium salt
Eqs.	Equation
ERCC1	Excision repair cross-complementation group 1
Fas	Fibroblast associated
FasL	Fas ligand
FCS	Fetal calf serum
Fig.	Figure

5-FU	5-Fluorouracil
GSH	Glutathione
GOI	Gene of interest
GTA	Graphite tube atomizer
HCT-8	Human ileocecal colorectal adenocarcinoma cell line
HCT-8ox	Oxaliplatin-resistant human ileocecal colorectal adenocarcinoma cell line
hCTR1	Human copper transporter 1
HMG	High-mobility group
hMLH1/2	Human mutL homolog 1/2
hOCT1-3	Human organic cation transporter 1-3
hMSH2/3/6	Human mutS homolog 2/3/6
hPMS2	Human postmeiotic segregation increased 2
HMG	High mobility group
ICP-MS	Inductively coupled plasma mass-spectrometry
ID	Inside diameter
IQR	Interquartile range
LLOQ	Lower limit of quantification
mal	Malonato
MAPK	Mitogen-activated protein kinase
MMR	Mismatch repair
mRNA	Messenger ribonucleic acid
MRP2	Multidrug resistance protein 2
MT	Metallothionein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
n.a.	Not applicable
n.s.	Not significant
NER	Nucleotide excision repair
NMR	Nuclear magnetic resonance
OAc	Acetato

OCT1-3	Organic cation transporter 1-3
OD	Outside diameter
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PSD	Programmable sample dispenser
p53	Tumor protein 53
p21	Cyclin-dependent kinase inhibitor 1A
p73	Tumor protein 73
PCR	Polymerase chain reaction
pEC_{50}	Negative logarithm (base 10) of EC_{50}
QC	Quality control
qRT-PCR	Quantitative real-time PCR
Pt	Platinum
RF	Resistance factor
RNA	Ribonucleic acid
RNase A	Ribonuclease A
RPA	Replication A
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SE	Standard error
SL	Stock solution
SLC	Solute carrier
S-phase	Synthesis phase
Tab.	Table
TCR	Transcription-coupled repair
TEA	Tetraethylammonium chloride
TFA	Trifluoroacetato
TMP	Thymidine 5'-monophosphate disodium salt
UBF	Upstream binding factor
UV/VIS	Ultraviolet/visible light
XPA	Xeroderma pigmentosum,

	complementation group A
XPF	Xeroderma pigmentosum, complementation group F
XPG	Xeroderma pigmentosum, complementation group G

1 Introduction

1.1 Antitumor platinum complexes

In 1844 Michele Peyrone synthesized cisplatin (cis-platinum(II)-diammine-dichloride, cDDP) without being aware of its tumor-inhibiting effect (Fig. 1-1). Not until the mid-1960s Barnett Rosenberg discovered the inhibition of cell division in the presence of this platinum complex. Due to its outstanding effectiveness in treating numerous tumors, cisplatin became the prototype for a new class of antineoplastic substances. Meanwhile it has become one of the most frequently used cytotoxic drugs in tumor therapy. However, its use is limited due to the side effects and the development of resistance, which has led to the search for new platinum complexes with anticancer properties[1].

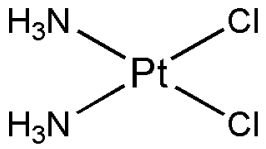

Fig. 1-1 Structure of cisplatin (cis-platinum(II)-diammine-dichloride).

Most of the antitumor platinum complexes developed up to now are uncharged cis-configurated square-planar platinum(II) complexes that can be described by the general formula cis-[PtA_2X_2], with A_2 as either two monodentate or one bidentate stable amine ligand(s) and X_2 as two monodentate or one bidentate anionic leaving ligand(s). Octahedral platinum(IV) complexes, which are now under development, can be described by the general formula cis-[$PtA_2X_2Y_2$] with Y_2 as monodentate anionic leaving ligands. The advantage of platinum(IV) complexes is the possibility of oral administration due to their increased stability and solubility in the gastrointestinal tract[1,2].

In the following chapters the processes taking place after the administration of cisplatin are described.

1.1.1 Mode of action

Bioactivation. After intravenous administration a high amount of cisplatin is bound to plasma proteins and thereby inactivated. Because of the high extracellular chloride concentration (≈ 100 mM), free cisplatin exhibits relatively low reactivity in the plasma. After entering the cell containing low chloride concentrations (≈ 4 mM) reactive monoaqua and diaqua complexes are formed by exchange of the chloride ligands. After activation, cisplatin is able to interact with a variety of macromolecules present in the cell[3].

Formation of platinum-DNA adducts. The cytotoxic effect is generally accepted to be mainly a consequence of the formation of platinum-DNA adducts. The primary target is the N7 position of the purine bases adenine and guanosine due to the high nucleophilicity of the imidazole ring. Cisplatin forms bifunctional 1,2- or 1,3-intrastrand crosslinks (60 – 65 % intrastrand GG, 25 – 30 % intrastrand AG, 5 – 10 % intrastrand GNG adducts), interstrand crosslinks (1 – 3 % interstrand GG adducts) and monofunctional adducts (Fig. 1-2)[4,5].

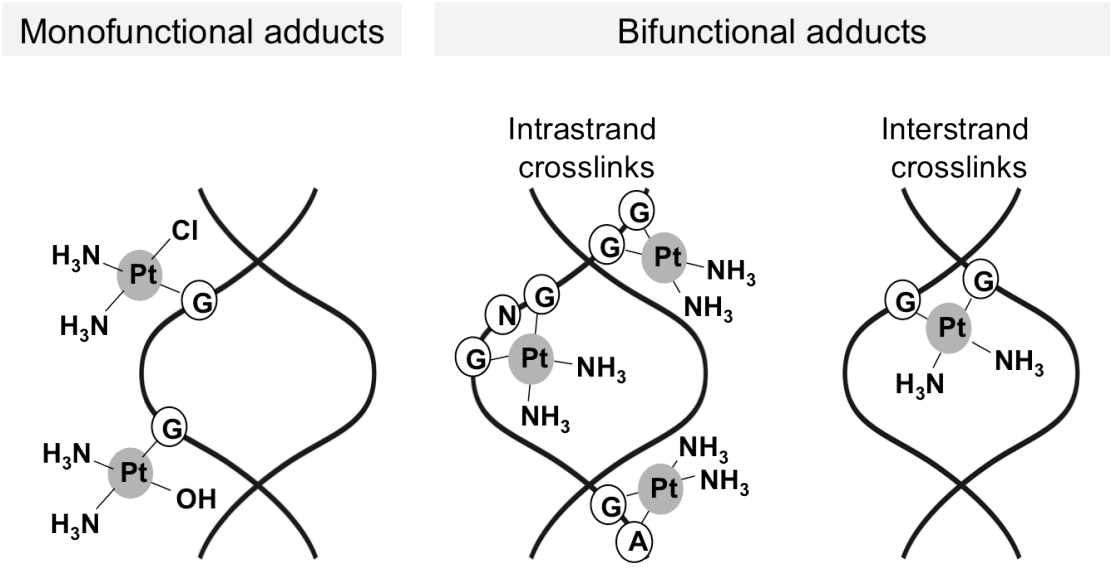

Fig. 1-2 Formation of platinum-DNA adducts. G, guanosine; A, adenosine; N, nucleoside.

1.1.2 Cellular response

Inhibition of DNA synthesis. As a result of the structural change of the DNA helix binding of DNA polymerases is hampered inhibiting replication and transcription[5].

DNA repair mechanisms. Platinum-DNA adducts are mainly repaired by nucleotide excision repair (NER) (Fig. 1-3)[5,6]. Due to small substrate specificity of this repair system, it is unlikely that platinum-DNA adducts of structurally different platinum complexes are differentiated[4,7,8]. A complete removal of the DNA damage is not possible in this way because of the limited capacity of the NER. Furthermore, adducts can be bypassed by some DNA polymerases. This translesion DNA synthesis – the so-called replicative bypass (post replicative repair) – allows the cells to progress through the S-phase (DNA replication) of the cell cycle. The mismatch repair (MMR) system is an important prerequisite for the cytotoxic activity of cisplatin. Base-pair mismatches are recognized and repaired by MMR proteins. The DNA strand previously synthesized is cut out and the DNA is again synthesized beyond the damaged sites entering in a vicious cycle. In the long term, these futile repair attempts lead to an induction of apoptosis. Thus, deficiency of MMR is associated with cisplatin resistance (see 1.1.3)[6].

Binding of HMG-proteins and transcription factors. The disturbance of the DNA structure results in binding of different proteins[5]. Among them high mobility group box proteins– such as HMGB1 – protect platinum-DNA adducts against repair proteins by stably binding to the platinum-DNA adducts. Many HMG box proteins are transcription factors and partly exhibit a higher affinity for the platinated DNA than for their natural substrate. The transcription factors are intercepted by the platinum-DNA adducts, therefore the transcription is inhibited. This phenomenon is called “transcription factor hijacking”[5].

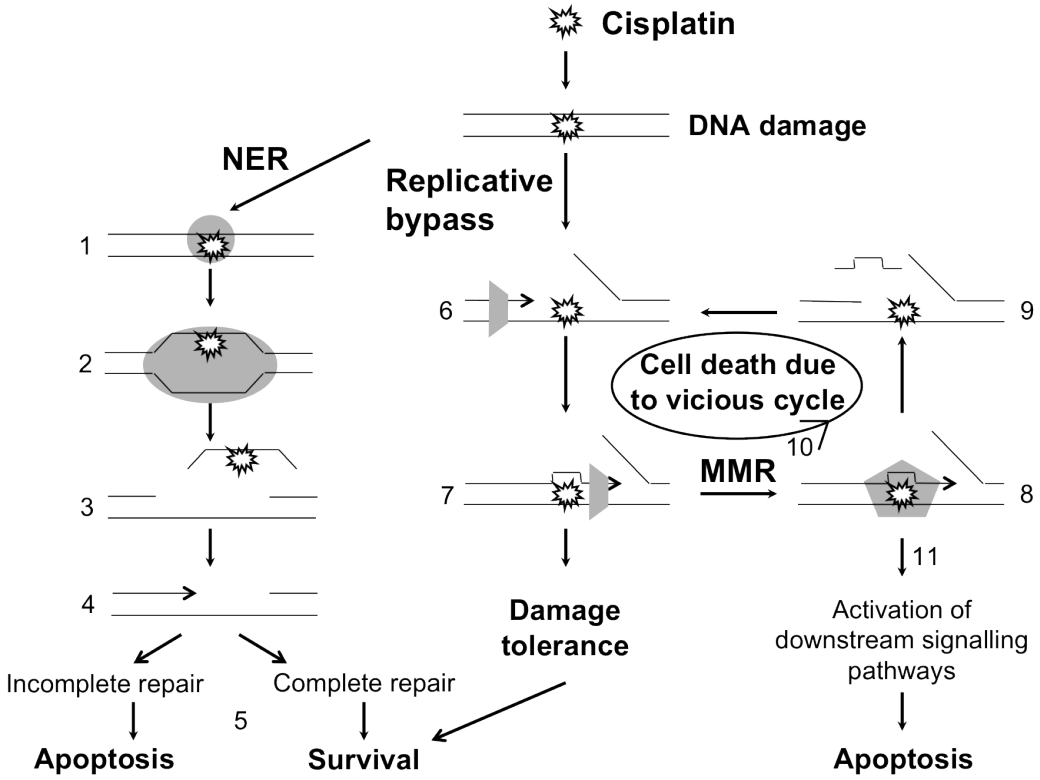

Fig. 1-3 DNA repair mechanisms. NER targets damaged or inappropriate bases within DNA. XPA and RPA are repair factors involved in the formation of a damage recognition subunit (1). This subunit triggers the recruitment of endonucleases (XPG and ERCC1-XPF) responsible for a dual incision at both 3´ and 5´ sites to the leasion (2). The damage-containing oligomers of 22 to 32 nucleotides in length are excised (3). Finally, DNA resynthesis and ligation of the new strand to the parental DNA takes place (4). If a complete repair is possible, the cell will survive. Otherwise, the cell undergoes apoptosis (5). During replicative bypass DNA repair enzymes carry on DNA synthesis despite the presence of platinum-DNA adducts on the parental strand (6 + 7). Consequently, no gaps or discontinuities into the newly synthesized strand occur and the cell survives. However, base-pairing errors are produced in the course of replicative bypass. Mismatch repair proteins, called Mut proteins (hMLH1, hMLH2, hPMS2, hMSH2, hMSH3, hMSH6), are able to recognize these mismatched DNA base pairs (8) and initiate the assembly of proteins which excise the affected area (9). Because MMR is directed to the newly synthesized strand, the platinum-DNA adduct remains at the parental strand. Therefore, replicative bypass may start again (6). The continued action of these futile excision and resynthesis cyles results in the formation of gaps or strand breaks, which lead to cell death (10). Another mechanism that induces apoptosis is the activation of downstream signalling pathways by the binding of the MMR proteins to the platinum-DNA adduct (11).

Induction of apoptosis and necrosis. The DNA damage caused by cisplatin triggers apoptosis and necrosis (Fig. 1-4). Among others, the following ways of apoptosis are being discussed[5]:

- The p53-dependent mitochondrial apoptosis with subsequent activation of caspase 9 and caspase 3.
- The Fas/Fas ligand (FasL)- and caspase-8-mediated apoptotic cascade.
- Activation of c-Abl tyrosine kinase and subsequent activation of p73 (p53 genes family).
- Mitogen-activated protein kinase (MAPK) pathways.

Necrosis occurs following excessive DNA damage, which induces hyperactivation of poly(ADP-ribose) polymerase (PARP). PARP causes ATP/NAD^+ depletion leading to necrotic cell death[5].

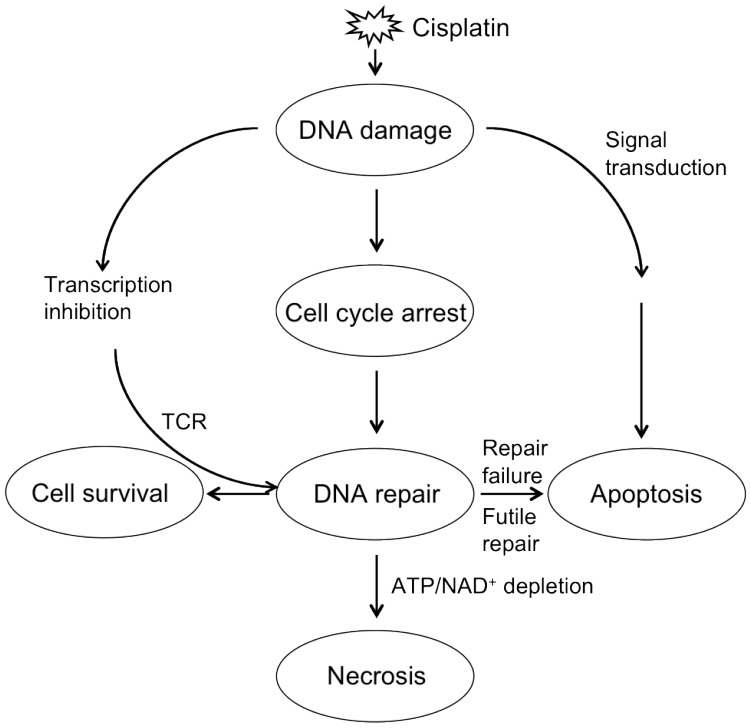

Fig. 1-4 Cell death pathways activated in response to cisplatin (reproduced from Wang and Lippard[5]). TCR, transcription-coupled repair.

1.1.3 Mechanisms of resistance

Resistance can be intrinsic or acquired by chronic drug exposure. Resistance to cisplatin is multifactorial, different resistance mechanisms may develop in parallel. Four major resistance mechanisms can be distinguished[3,5,6].

Reduced accumulation. After exposure to platinum complexes, a decreased influx, an increased efflux, or a combination of both phenomena was observed. Previously, it has been assumed that platinum complexes enter the cell primarily by passive diffusion (Fig. 1-5). During the last decade a link between copper transporters (CTR1, ATP7A, ATP7B) and platinum influx and efflux has been discovered in numerous studies. Recent results furthermore indicate an involvement of organic cation transporters (OCT1-3). Platinum resistance may develop by upregulation of efflux or downregulation of influx transporters.

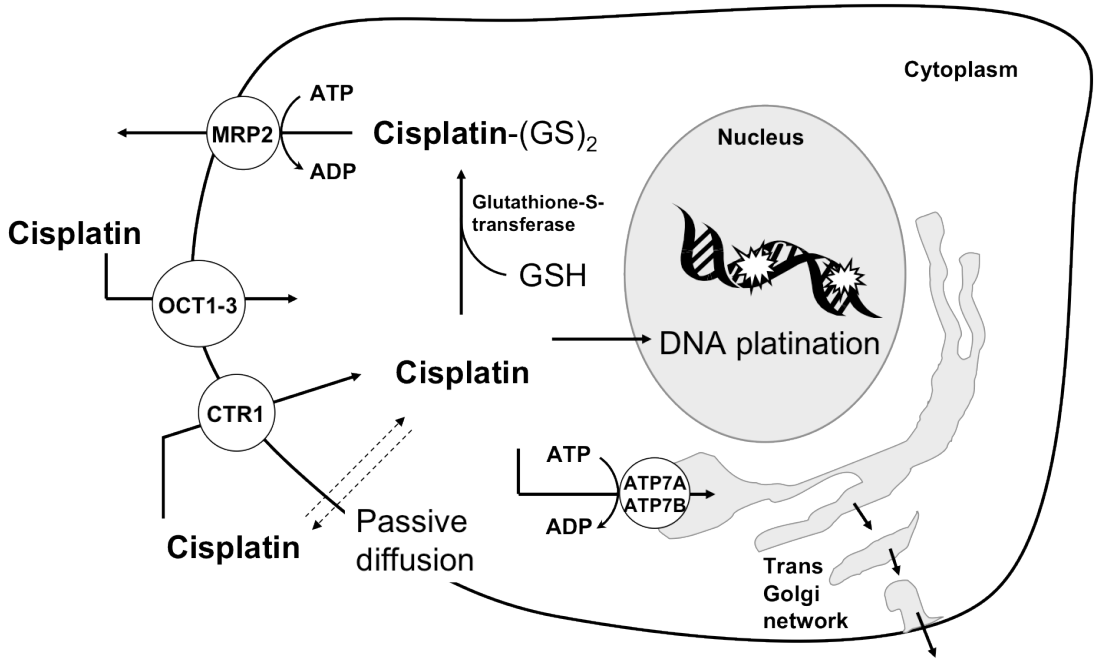

Fig. 1-5 Mechanisms of cisplatin influx, efflux and detoxification. ATP7A, adenosine triphosphate alpha-polypeptide; ATP7B, adenosine triphosphate beta-polypeptide; CTR1, copper transporter 1; MRP2, multidrug resistance protein 2; OCT1-3, organic cation transporters 1-3.

Increased inactivation. Platinum complexes react to a great extent with intracellular molecules that contain thiol groups, e.g., glutathione (GSH) or metallothionein (MT). Only a relatively small fraction of intracellular cisplatin binds to genomic DNA. It has been estimated that only 1 % of the platinum that enters the cell binds to nuclear DNA[9]. According to prior studies more than 60% is bound to GSH[10]. However, recent results suggest that the fraction of platinum(II) bound to GSH is of maximum 20 %[11]. The platinum-GSH conjugate is biologically inactive and can be removed from the cell by the transporter MRP2 (Fig. 1-5)[12]. In addition, the formation of interstrand crosslinks can be prevented by interaction between thiol-containing molecules and monofunctional adducts. Therefore, an increased intracellular GSH production can contribute to the development of resistance[5,6,13].

Increased adduct tolerance and failure of apoptotic pathways. An increase of replicative bypass leads to an increased adduct tolerance as well as to a high mutation rate, whereby changes, which result in subsequent development of resistance, are favored. Defects in the MMR system prevent the beginning of futile repair cycles, hence contributing to resistance[5]. The complex cascades of apoptosis offer a further starting point for the development of resistance. Many factors can be changed, examples are the loss of p53 function, reduced activity of caspases, or a deregulated MAPK pathway[5].

Increased repair. A general resistance mechanism is increased repair by the NER system. The capacity of this kind of resistance mechanism seems, however, to be limited[5].

1.1.4 Clinical relevance

Meanwhile platinum compounds are used in 50 % of all tumor therapies[14]. A selection of the EU-approved indications of cisplatin, carboplatin and oxaliplatin is presented in Tab. 1-1[15-17]. The addition of all approved indications leads to not less than 26 different indications of cisplatin, three of carboplatin and five different indications of oxaliplatin[18-20].

Tab. 1-1 Selection of approved indications of cisplatin, carboplatin and oxaliplatin with a focus on those indications approved via the decentralised and mutual-recognition procedure in EU countries.

Platinum compound	***Indication***
Cisplatin	Advanced or metastatic tumor of testis (palliative and curative therapy)
	Advanced or metastatic ovarian tumor
	Advanced or metastatic small cell lung carcinoma and non-small cell lung carcinoma
	Advanced or metastatic head and neck cancer (palliative therapy)
Carboplatin	Advanced epithelial ovarian cancer (first- and second-line)
	Small cell lung carcinoma
Oxaliplatin	Stage III colon cancer in patients who have undergone complete resection of the primary tumor (in combination with 5-fluorouracil (5-FU) and folinic acid; adjuvant therapy)
	Metastatic colorectal cancer (in combination with 5-FU and folinate)

The greatest success of antitumor platinum complexes was achieved by cisplatin in the treatment of testicular cancer. The response rate increased from a historical control of 25 % to approximately 90 % in patients with good prognosis[21]. Another milestone in cancer chemotherapy was the approval of oxaliplatin. Oxaliplatin is effective in the treatment of metastatic colon/colorectal carcinoma, which exhibit intrinsic resistance to cisplatin and carboplatin[22,23].

However, the treatment is limited by a high rate of severe adverse effects. Although the incidence of severe adverse effects could be limited, e.g. by hydration for prevention of nephrotoxicity and using serotonine receptor antagonists (5-HT_3),

dexamethasone and/or NK_1 receptor antagonists for antiemetic prophylaxis, they still remain a problem in the treatment with platinum compounds[24,25]. Relevant and dose-limiting adverse effects of cisplatin are nephrotoxicity, ototoxicity, emesis, and neurotoxicity[18,22,25,26]. The only dose-limiting adverse effect of carboplatin is myelosuppression. Among the severe adverse effects are imbalance of electrolytes, peripheral neuropathy and less-common visual disturbance[22,26,27]. The crucial and dose-limiting toxicity of oxaliplatin is peripheral sensory neuropathy[28]. Myelosuppression is low. Neither ototoxicity nor nephrotoxicity has been observed[20,22,26].

A further significant limitation toward the successful treatment of malignant cancers with platinum complexes is the emergence of drug resistance. It is hardly possible to quantify the degree of resistance, because a relapse of a patient might not only be due to resistance of tumor cells, but also due to rapid progression of the disease, characteristics of the drug (e.g., pharmacokinetics) and specific features of the patients (e.g., genetic polymorphisms)[29,30]. In case of advanced ovarian cancer a tumor is classified as platinum-resistant, if the treatment free interval is less than six months[31]. Almost all patients with advanced ovarian cancer at diagnosis relapse despite surgery and first-line therapy. With each relapse the response rate decreases[31]. The median progression-free survival is only 18 months and nearly all patients die of the disease[30,31]. In colorectal cancer the median progression-free survival increased from 12 to 22 months with the introduction of oxaliplatin and irinotecan[32]. Despite this amelioration, according to the Statistisches Bundesamt Deutschland a malignant neoplasm of the colon was still the ninth most frequent cause of death in Germany in 2008 with 17,920 deaths in total (2.1 %)[33].

To further improve platinum-based chemotherapy the underlying mechanisms of the development of toxicity and resistance have to be investigated in detail. Based on that knowledge supportive therapy might be improved and it might become possible to predict which patients will respond to platinum-based therapy. Because it is nearly impossible to conduct this kind of investigations in patients, many *in vitro* and some *in vivo* (e.g., knockout mice) models have been developed to elucidate the basic molecular principles of toxicity and resistance. Considerable process has been made

within the last years. Until today, the relevance of these findings could be only partly confirmed in patients[29,30].

An influence of different transporters on degree of toxicity was already demonstrated in *in vivo* models. For example, there is some evidence that the damage of outer hair cells of the cochlea resulting in ototoxicity is mediated by OCT2[34]. Concomitant administration of imatinib, which inhibits the influx of cisplatin in human embryonic kidney cells via OCT2, and cisplatin decreased cellular accumulation of platinum and subsequent nephrotoxicity in rats[35]. Furthermore, the transporter CTR1 is involved in neurotoxicity. The degree of cell body atrophy of CTR1-positive and -negative rat dorsal root ganglia after administration of platinum drugs was compared leading to the result that CTR1-positive rat dorsal root ganglia were vulnerable to toxicity of platinum compounds, especially to oxaliplatin[36].

In spite of intensive efforts of the scientists, the clinical relevance of the mechanisms described at the cellular level regarding the influx mediated by transporters and the contribution of transporters to resistance still needs to be defined. To date, only limited data are available. For instance, the role of the copper efflux protein ATP7B was demonstrated *in vivo*. Patients whose carcinomas expressed high levels of ATP7B had a significantly poorer prognosis than patients with tumors that expressed low levels of ATP7B[37-39]. Also, ovarian tumor patients with increased levels of ATP7A had a lower survival rate than those without increased levels of ATP7A[40]. However, the findings on the cellular level, could not always be confirmed in patients. Thus, for example, the expression of OCT2 in ovarian cancer did not correlate with treatment outcome, although an influence of OCT2 on the influx of cisplatin was demonstrated at the cellular level[41].

In conclusion, there is a great need for large prospective clinical trials to confirm the findings on the cellular level *in vivo*. A further investigation of *in vivo* toxicity and resistance mechanisms should be valuable in reducing the toxicity of platinum-based chemotherapy and in allowing the prediction of clinical response to chemotherapy. Additionally, new treatments should be identified with a lower profile of toxicity and with improved outcome in platinum-resistant tumors.

1.2 Pharmacokinetics and pharmacodynamics of oxaliplatin

To date, the platinum complexes cisplatin, carboplatin and oxaliplatin have gained worldwide approval (Fig. 1-6). The third-generation drug oxaliplatin shows an improved therapeutic profile compared to cisplatin and carboplatin. First, oxaliplatin produces cytotoxicity in platinum-resistant cell lines[7,8]. Furthermore, oxaliplatin has a better safety profile than cisplatin and carboplatin, because it lacks nephrotoxicity observed with cisplatin, produces less myelosuppression than carboplatin and patients experience only mild nausea/vomiting during treatment[23,42,43]. The superiority of oxaliplatin is based on changes in the molecular mechanisms. To elucidate the reasons for the superiority of oxaliplatin, the molecular mechanisms of oxaliplatin are described in the present chapter, with particular reference to influx as the main topic of this thesis.

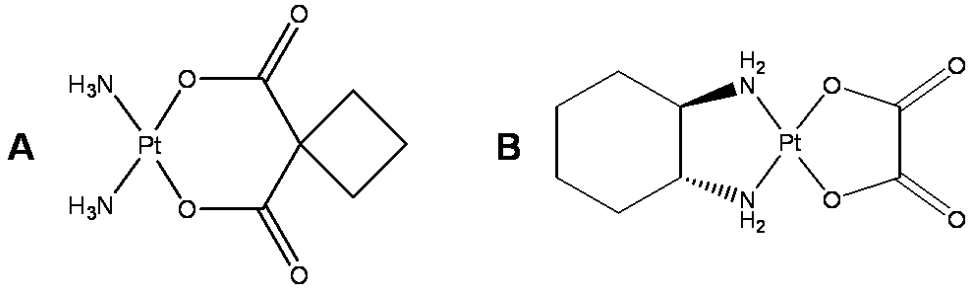

*Fig. 1-6 Structure of **A** carboplatin and **B** oxaliplatin.*

1.2.1 Extracellular biotransformation

After a 2 h infusion of oxaliplatin, 15 % of the administered platinum is present in the blood. The remaining 85 % has undergone distribution from the plasma into tissues or has been subjected to urinary elimination. 40 % of the platinum in blood is irreversibly bound to erythrocytes, about 30 % is bound to plasma proteins and the rest remains unbound[44,45]. The unbound fraction is supposed to contain the intact oxaliplatin and its biotransformation products.

Biotransformation of oxaliplatin occurs rapidly and is nonenzymatic[44]. Several low molecular weight biotransformation products from reactions with water, chloride, glutathione, methionine and cysteine as well as unidentified products have been

observed *in vitro* and *in vivo*[46-53]. However, appearance and importance of these products are still a matter of discussion. In a pharmacokinetic study of oxaliplatin in Wistar rats following a bolus injection (20 µmol/kg), the plasma AUC was 30-fold less and c_{max} was 50-fold lower for $Pt(DACH)Cl_2$ than for oxaliplatin[50]. The maximum plasma concentrations for $Pt(DACH)(H_20)Cl$ and $Pt(DACH)(H_2O)_2$ are at least 1,000-fold lower than that for oxaliplatin. The low plasma concentrations of these biotransformation products might be explained by the low probability of $Pt(DACH)Cl_2$ to be hydrolyzed into aquated species to a significant extent in presence of the high plasma concentration of chloride ions (≈ 100 mM)[50]. However, this explanation only holds, if the formation of the chloro complex is a mandatory step prior to the formation of the aquated species, but this is not of necessity[45,49]. Recently, Ip et al. investigated the concentrations of oxaliplatin and $Pt(DACH)Cl_2$ in plasma of colorectal cancer patients during (2 h of infusion, 130 mg/m^2) and after infusion (up to 2 h)[48]. During infusion 58 % (1 h) and 63 % (2 h) of total platinum in plasma accounted for oxaliplatin. At the end of infusion intact oxaliplatin decreased rapidly ($t_{1/2}$ = 0.25 h). $Pt(DACH)Cl_2$ was not detected in the plasma. The authors proposed that the appearance of the chloro complex in the earlier studies was an artefact of sample preparation or storage procedures[48].

Against the background of oxaliplatin influx, these investigations are of high relevance, because until now it was suggested that oxaliplatin enters the cell as a dichloro complex. The absence of the dichloro complex *in vivo* excludes a contribution of this complex to influx. The high fraction of intact oxaliplatin in the plasma could make one conclude that the cell takes up intact oxaliplatin. However, given the dynamic nature of the biotransformation and the compartmentalization of drug, it is still possible that a number of different species enter the cell by different routes with varying kinetics.

1.2.2 Influx

Gately and Howell postulated the influx of platinum complexes to be mediated by passive diffusion and by facilitated diffusion through a gated channel[54]. Later this model was broadened to include not only gated channels but facilitated and active transport mechanisms involving a number of transport proteins[3]. The contribution of

the copper transporter CTR1 and the organic cation transporters OCT1-3 to oxaliplatin influx is widely discussed[3,55].

1.2.2.1 Passive diffusion

Characteristics. It is generally thought that many molecules are transported across biological membranes via passive diffusion. Passive diffusion occurs down a concentration gradient from high to low concentration. The concentration gradient disappears as diffusion proceeds. Therefore, the rate of diffusion is proportional to the difference in solute concentration on the inner and outer side of the membrane (Fick's first law). Furthermore, the rate of diffusion mainly depends on size, lipophilicity and charge of the molecule. In general, passive diffusion of small, lipophilic and uncharged molecules is fast[56].

Role in influx of oxaliplatin. Platinum complexes are suggested to enter cells via passive diffusion[3]. This was not only demonstrated for cisplatin, but also for oxaliplatin. Mishima et al. showed that influx of oxaliplatin in five cell line pairs was neither saturated by time nor by concentration[57].

1.2.2.2 Copper transporter 1 (CTR1)

Characteristics. The copper transporter 1 (CTR1) belongs to the solute carrier family SLC31[58]. CTR1 is a high affinity copper transporter, which mediates cellular influx of copper. It is suggested to be Cu(I)-specific, i.e. either prior to or concomitant with the influx, Cu(II) is reduced to Cu(I) by one or more metalloreductases[59]. Once inside the cell, copper is distributed to cellular proteins and compartments for the incorporation into copper-dependent proteins[60]. CTR1 consists of three transmembrane domains (190 amino acids), a methionine-rich N-terminus (M7XMXXM, M40MMMXM), a cysteine-histidine cluster in the C terminus and an MX_3M motif in the second transmembrane domain (M150XXXM) (Fig. 1-7)[61,62]. The transporter is present in the membrane as a homotrimer that is likely to act as a pore between the subunit interfaces[63,64]. The pore involves a series of methionine rings (M150 and M154) each containing three methionines and a ring of three cysteines (C189)[64]. The pore diameter is ≈ 8 Å at its narrow end towards the extracellular exit

and ≈ 22 Å towards the intracellular end[64]. The passage of copper seems to be associated with conformational changes in CTR1[64]. However, the details of the transport of copper across the membrane are still unknown[61,65]. The MX_3M motif is essential for function of copper influx. Regarding the contribution of the N-terminal methionine-rich domain the results are contradictory. While Puig et al. demonstrated that this domain is only necessary under copper-depleting conditions, Liang et al. found that it is also essential for function of copper influx[62,66]. The extracellular methionine-rich domain is not involved in the process of oligomerization, because its deletion did not affect the ability of oligomerization, however, resulted in cytoplasmic localization of the transporter[62].

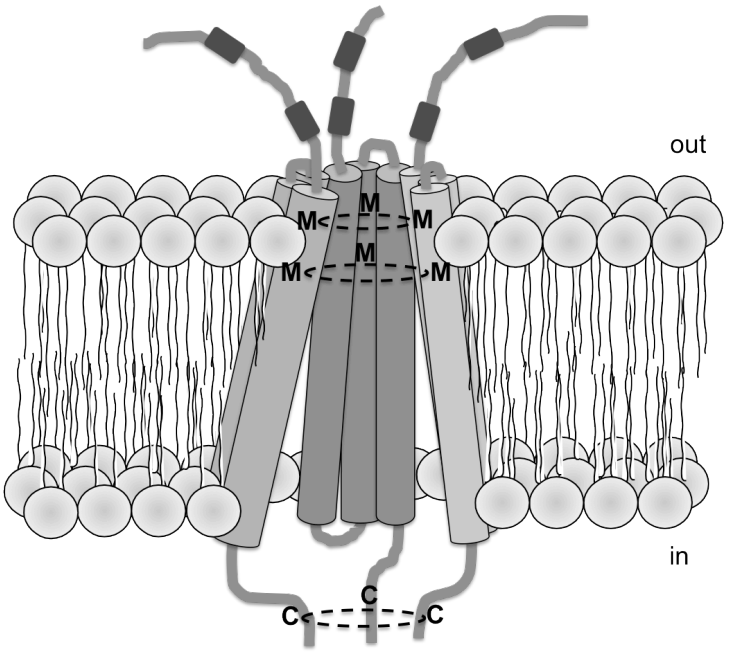

Fig. 1-7 A model for human copper transporter 1 (hCTR1) in the phospholipid bilayer. A hCTR1 monomer consists of 190 amino acids. The N terminus projects outside and the C terminus inside the cell. The extracellular domain has two methionine-rich domains (▬). Three hCTR1 monomers oligomerize to form a pore. The trimeric pore provides a series of stacked rings of methionines (M) and cysteines (C) that might participate in transchelation reactions (modified from Howell et al.[65]).

Role in oxaliplatin influx. The copper transporter 1 (CTR1) appears to contribute to influx of oxaliplatin, as could be demonstrated by an impaired accumulation of oxaliplatin in CTR1-deficient cells[55,67,68]. Because the influx was dependent on CTR1

only at low oxaliplatin concentrations, oxaliplatin has been concluded to be a substrate for some other mechanism of cellular entry[67]. A further evidence for the contribution of CTR1 to oxaliplatin influx was provided using CTR1-positive and -negative neurons of rat dorsal root ganglia[69]. CTR1-positive neurons became atrophied after treatment with oxaliplatin, whereas CTR1-negative neurons showed less treatment-induced cell body atrophy[69]. Unfortunately, information about the mechanisms of influx of the drug via CTR1 is still limited. Most of the investigations have been carried out with respect to cisplatin. Nevertheless some general considerations are as follows:

- The size of the pore (≈ 8 Å) may pose an obstacle for the passage of oxaliplatin (≈ 4 x 4 x 10 Å)[65,70]. However, the protein may be quite flexible.
- Oxaliplatin as a soft Lewis acid forms weak bonds with one or more methionine(s). Therefore, it is conceivable, that oxaliplatin undergoes transchelation reaction from one methionine to the next. The molecular distance between the methionine rings formed by M154 and M150 is short enough to allow this reaction[65].

Influence on cytotoxicity of oxaliplatin. The crucial question, if the impaired or increased accumulation of oxaliplatin influences cytotoxicity, was also investigated. Regarding CTR1, a deletion of both alleles did not affect the growth inhibitory effect of oxaliplatin[67]. However, the concentrations used in the influx studies were not comparable with the concentrations used in the cytotoxicity studies[67].

Influence on resistance to oxaliplatin. The contribution of the transporter to resistance has been less investigated and the results are contradictory. Up- and downregulation of hCTR1 or even no changes in the expression of hCTR1 in different platinum-resistant cell lines were observed[71-74]. An explanation of these discrepancies might be, that CTR1 is not functional in some resistant cell lines, although it is expressed at a normal level[65]. This might be possible due to the failure of resistant cells to glycosylate CTR1 at T27. Consequently, the protein renders susceptible to proteases that excise the N-terminal domain and inactivate the transport function of CTR1 while leaving the rest of the protein in the membrane[75].

1.2.2.3 Organic cation transporters (OCT1-3)

Characteristics. The organic cation transporters belong to the SLC22 family encompassing not only the cation and carnitine transporters (OCTN1, OCTN2, OCT6) and the proton/cation antiporters (MATE1, MATE2-K, MATE2-B), but also the polyspecific cation transporters OCT1-3 (SLC22A1-3)[76,77]. OCT1 and OCT3 exhibit a broad tissue distribution including intestine, liver, kidney, lung, skin, epithelial and tumor cells. In contrast to OCT1 and OCT3, the tissue expression pattern of OCT2 is more restricted. It is strongly expressed in the kidney, however, also found in a variety of other organs[76,77]. The organic cation transporters contain 12 transmembrane domains with one hydrophilic loop connecting the first and the second transmembrane domaine assigned to the extracellular side. Another hydrophilic loop is localized intracellular connecting the sixth and seventh transmembrane domaine (Fig. 1-8)[76].

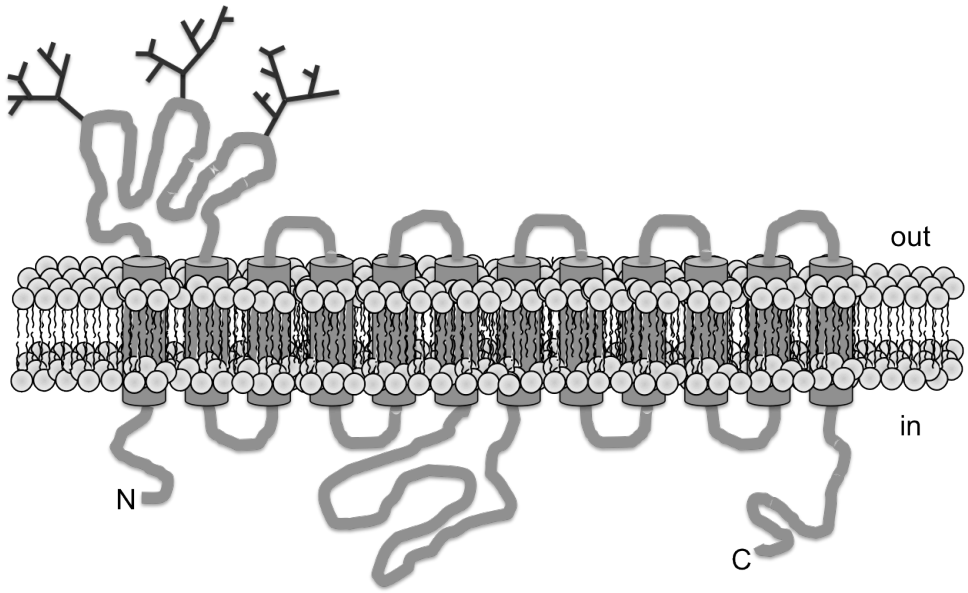

Fig. 1-8 Predicted structure of hOCT1-3. N and C terminus are located intracellularly (in). The large extracellular loop between the first and the second transmembrane domain contains three N-glycosylation sites.

Not only organic cations and weak bases that are positively charged at physiological pH, but also non-charged molecules are transported in an electrogenic manner. The transport across the plasma membrane is independent of sodium and proton

gradients and occurs in both directions. Transported substrates exhibit mutual inhibition, which may be total or partial. OCTs translocate a variety of molecules and numerous molecules are able to inhibit this transport. The question, which kind of properties a molecule has to possess to act either as a substrate or as an inhibitor, is difficult to answer, because the affinity of transported substrates and non-transported inhibitors overlap broadly[77]. In recent studies identifying molecular determinants of substrate/inhibitor binding to OCTs, pharmacophores for OCTs have been reported[78-85]. Hydrophobicity and basicity are important determinants of substrate specificity for OCTs[84]. Three pharmacophores have been reported for OCT1. The first one consists of three hydrophobic sites and a positive ionizable site[79]. The alternative pharmacophores consist of a positive ion interaction site, a hydrophobic interaction site, and one or two hydrogen bond acceptor sites, whereas the calculated distance between the hydrophobic and hydrogen bond acceptor sites was between 4 and 7 Å[79-82]. The two-point pharmacophore for OCT2 suggested by Zolk et al. consists of an ion interaction site and a hydrophobic aromatic site separated by 5 Å[85]. The only physicochemical descriptor identified was the topological polar surface area (TPSA). The smaller the TPSA values of a drug, the higher was its inhibitory effect on the transport of model cations. An influence of the log P value, pK_a value, log S value (aqueous solubility) and solvent accessible surface area (SASA) on inhibition of the transport of the model cations could not be demonstrated[85]. However, Suhre et al. found an influence of hydrophobicity on the OCT2-drug interaction[83]. Contrary to OCT1 and OCT2, information on OCT3-drug interaction is still limited.

Role in oxaliplatin influx. Recent results suggested that human organic cation transporters (hOCT1-3) may play an important role in the influx of oxaliplatin[41,86-89]. In different cell systems a higher accumulation of oxaliplatin in hOCT1-, hOCT2- and/or hOCT3-transfected cells was observed[41,87,88,90]. Only one study failed to demonstrate an involvement of hOCT2 in the transport of oxaliplatin[91].

Influence on cytotoxicity. The crucial question, if the impaired or increased accumulation of oxaliplatin influences cytotoxicity, was also investigated. In most of the hOCT1-3-transfected cell lines a higher oxaliplatin accumulation led to a higher cytotoxicity[41,87,88,90].

Influence on resistance. The contribution of the transporters to resistance has been less investigated and the results are contradictory. Upregulation of hOCT1 and hOCT2, as well as downregulation of hOCT3 were observed in the resistant cell line[73].

1.2.3 Intracellular biotransformation

Once inside the cell, the activation mechanism of oxaliplatin is unclear. The oxalate leaving group might be displaced by chloride under formation of $Pt(DACH)Cl_2$[92]. However, it was shown that the percentage of $Pt(DACH)Cl_2$ in the cytosolic ultrafiltrate after incubation with oxaliplatin is similar to that observed in the culture medium. This implies that the intracellular conversion of oxaliplatin to $Pt(DACH)Cl_2$ is low[92]. Consistent with these results, Mauldin et al. demonstrated the intracellular displacement of the malonato leaving group in the oxaliplatin analogue Pt(DACH)(mal) with chloride to be very slow (< 5 % in 24 h)[93]. Moreover, they suggested an activation of platinum complexes with bidentate leaving groups by the intermediate formation of highly reactive bicarbonato or phosphato complexes[93,94]. But the low reactivity of oxaliplatin with bicarbonate or phosphate under physiological conditions is in disagreement with this hypothesis[45].

Other important reactants in the intracellular environment are sulfur-containing nucleophiles. Oxaliplatin exhibits a higher reactivity towards sulfur-containing compounds than cisplatin[95]. The reaction rate with glutathione and cysteine was about six times higher for oxaliplatin, whereas the rate with methionine was only about twice as fast for oxaliplatin as compared to cisplatin. At the intracellular concentration of sulfhydryl groups up to 10 mM a half-live of oxaliplatin of less than 15 min was estimated[95]. The fate of the platinum complexes bound to sulfur is largely unknown. The complexes might be inactive or serve as drug reservoirs[10,96]. However, it has to be taken into account that the experiments have not been conducted in the intracellular environment.

1.2.4 Efflux

The copper transporters ATP7A and ATP7B might contribute to the cellular efflux of oxaliplatin. An increased expression of ATP7A in ovarian cancer cells rendered cells resistant to oxaliplatin[97]. However, cellular accumulation was not reduced as expected, but an enhanced sequestration of the drug into the vesicular fraction was shown. It was suggested that ATP7A sequesters oxaliplatin and keeps it thereby away from the DNA[97]. Furthermore, some other studies also point at the contribution of ATP7A and ATP7B to oxaliplatin resistance due to an increased efflux or sequestration[38,74]. ATP7A was overexpressed in response to oxaliplatin exposure in sensitive and oxaliplatin-resistant cells[74]. Analysis of tumors from colorectal cancer patients treated with oxaliplatin/5-FU revealed that patients with lowest mRNA expression levels of ATP7B had significantly longer time to progression than patients with highest levels[38]. Nevertheless, the results are cell line specific. Fibroblasts transfected with ATP7A or ATP7B were hypersensitive to oxaliplatin[98]. The fact, that oxaliplatin-resistant cells exhibited only basal expression of ATP7A and ATP7B compared to their sensitive counterparts also point at the cell line specifity of the results[74].

A contribution of two members of the multidrug and toxin extrusion family (MATE1 and MATE2-K) to the efflux of oxaliplatin in renal proximal tubular cells has also been reported[99]. hMATE1 and in particular hMATE2-K can mediate the H^+-dependent anti-port of oxaliplatin. This mechanism of efflux mainly plays a role in renal cells, because these transporters are primarily expressed in the kidney.

1.2.5 Mechanism of action

Oxaliplatin shows another spectrum of efficacy and resistance than cisplatin and carboplatin. The oxalate ligand as the leaving group leads to a slightly reduced reactivity compared to cisplatin. The bulky 1,2-diaminocyclohexane (DACH) ligand is probably responsible for the improved water solubility as well as the altered profile of activity[7,100]. Using equimolar and equitoxic concentrations of cisplatin and oxaliplatin, it was found that the extent of DNA platination is substantially lower after treatment with oxaliplatin[7,8,101]. However, the rate of single-strand breaks is higher[8]. It is still

unknown, whether these single-strand breaks are induced as a result of primary DNA lesions or are due to rapid induction of apoptosis[8].

The activity profiles of oxaliplatin and cisplatin might be different, because mismatch repair proteins, damage-recognition proteins and translesion DNA polymerases differentiate between cisplatin- and oxaliplatin-DNA adducts[4].

Mismatch repair proteins. The DACH ligand reduces the affinity of MMR proteins for oxaliplatin-DNA adducts[4,7,8]. However, this does not reduce the cytotoxic effect of oxaliplatin, to wit, the cytotoxic effect of oxaliplatin is independent of the MMR system. This enables oxaliplatin to be active in MMR-deficient cells, which are resistant to cisplatin, and might be the reason for the absence of cross-resistance to cisplatin[4,7,8].

Damage-recognition proteins. HMG box proteins (e.g. HMG1, HMG2, Ixr1, tsHMG, SRY, LEF-1, hUBF) and non-HMG box proteins (e.g. TBP, histone H1, p53, XPE, Ku, XPA, RPA) bind with high affinity to platinum-DNA adducts (see 1.1.1). A difference in the affinity of these proteins in binding to cisplatin-DNA adducts compared to oxaliplatin-DNA could be demonstrated. Especially HMG box proteins bind more tightly to cisplatin-DNA adducts than to oxaliplatin-DNA adducts. However, the biological consequences of the different binding behavior are unclear[4,7].

Translesion DNA polymerases. Polymerases are involved in the translesion synthesis of platinum-GG intrastrand adducts and are able to discriminate between cisplatin- and oxaliplatin-DNA adducts[4]. Of the human polymerases tested in previous studies, both pol η and pol β bypass oxaliplatin-GG adducts with higher efficiency than cisplatin-GG adducts. Both polymerases are suggested to mediate error-free bypass of lesions. The results of the study of Yang et al. are consistent with this suggestion[102]. They observed that cells deficient of pol β were hypersensitive to oxaliplatin treatment[102]. Another translesion DNA polymerase, pol ζ, also discriminates between cisplatin- and oxaliplatin-DNA adducts. However, contrary to pol η and pol β, pol ζ is involved in error-prone translesion synthesis. It was shown that loss of pol ζ confers greater sensitivity to cisplatin than to oxaliplatin[4].

1.3 Overcoming platinum resistance by chemical optimization

As outlined in chapter 1.1.4, toxicity and resistance of cancer cells to platinum-based drugs remain a significant impediment to successful chemotherapy. Nevertheless, antitumor platinum compounds exhibit a broad spectrum of activity and high antitumor activity. Therefore, efforts have been ongoing to design new antitumor platinum compounds with

- superior efficacy
- reduced toxicity
- lack of cross-resistance
- and improved pharmacological properties compared to the parent compound, cisplatin, e.g., providing oral bioavailability.

However, after the introduction of cisplatin in chemotherapy only two platinum complexes have received worldwide approval: carboplatin and oxaliplatin. Carboplatin differs from cisplatin by the nature of the leaving group, cyclobutan-1,1-dicarboxylate instead of two chloride moieties. The new leaving group leads to increased water solubility and stability compared to cisplatin. Toxicity was decreased. However, cisplatin and carboplatin show cross-resistance, which points to a similar mechanism of action[100]. Oxaliplatin shows another spectrum of efficacy and resistance. The oxalate ligand as the leaving group leads to a slightly reduced reactivity compared to cisplatin[100]. The bulky 1,2-diaminocyclohexane (DACH) ligand is probably responsible for the reduced water solubility as well as the changed profile of activity[23].

The differences in toxicity and anticancer activity between the above-mentioned approved platinum complexes can be explained by structure-activity relationships. The leaving group of a platinum compound largely determines its reactivity[103]. Reactivity is defined as the ability to exchange the leaving group(s) upon interaction with nucleophiles. In the case of cisplatin and carboplatin, which only differ in the structure of their leaving group, one would expect differences in reactivity. In fact, *in vitro* determination of reactivity towards calf thymus DNA showed higher reactivity of cisplatin than of carboplatin[104]. It could be demonstrated that equal binding was

followed by equal cytotoxicity. Therefore, once the compounds are bound to DNA, they exhibit equal cytotoxicity. However, to achieve equal binding the dose of carboplatin has to be 20 to 40-fold higher than that of cisplatin which points at an altered biodistribution of carboplatin[104,105]. A positive effect of the reduced reactivity is the advantageous toxicological profile of carboplatin[106,107]. In general, the stability of a platinum complex regarding leaving group(s) can be increased by introduction of chelated, dicarboxylate-containing moieties with the reactivity decreasing in the order oxalate > malonate > phenylmalonate > cyclobutan-1,1-dicarboxylate[100]. Inert platinum complexes usually exhibit low cytotoxic potency and low toxicity, whereas reactive platinum complexes are often of high potency and toxicity[103,105].

It is believed that reactivity also influences platinum influx[67,108-110]. Pereira-Maia et al. investigated the relationship between the concentration of the extracellular cisplatin-aquated species and the rate of cellular influx[109]. It was hypothesized that only the aquated species were transported by an active influx mechanism. More reactive platinum complexes, like cisplatin, produce higher amounts of aquated species. Therefore, they are taken up faster than less reactive platinum complexes, like carboplatin[109]. However, the hypothesis that the first step in influx of cisplatin is the formation of the aquated species in a Cl^--deficient space at the level of the plasma membrane is in disagreement with the knowledge about the bioactivation of cisplatin, because according to the results of the previous studies the formation of the aquated species takes place not before cisplatin has entered the cell (see 1.1.1)[3]. In another study, a relationship between reactivity of different diammine/diaminocyclohexane platinum(II) complexes and cellular influx rates could be demonstrated[110]. However, in this case reactivity was determined based on interaction with G-actin, which is not likely to be relevant in vivo, and influx experiments were carried out in human erythrocytes[110]. Furthermore, the relevance of reactivity for influx mediated by the human copper transporter 1 (hCTR1) has been discussed. It has been suggested that platinum complexes stabilize a homotrimer of hCTR1 by cross-linking hCTR1 subunits via methionine-rich clusters[108]. The extent of the formation of the homotrimer depended on the reactivity of the platinum complex. Consequently, trimerization was less efficient after treatment with oxaliplatin compared to cisplatin and even slower after treatment with carboplatin[108]. Nevertheless, besides reactivity, which seems to be important for binding to methionine-rich clusters, the bulky

diaminocyclohexane amine ligand was suggested to mediate the influx of oxaliplatin by a CTR1-independent mechanism[67].

It should be mentioned that not only the leaving group determines the reactivity of platinum complexes but also the carrier ligand is able to modify the reactivity, in an indirect manner, by steric, electronic and basicity effects[103,111]. Furthermore, the carrier ligand determines the structure of the platinum-DNA adduct. Because cisplatin and carboplatin form the same platinum-DNA adducts, their sensitivity and resistance pattern is similar[4]. However, platinum-DNA adducts formed by oxaliplatin are bulkier and more hydrophobic[13]. The different structure of the platinum-DNA adducts appears to contribute to the lack of cross-resistance with cisplatin and carboplatin[5]. Comparable to oxaliplatin a circumvention of cisplatin resistance could also be demonstrated with carboplatin analogues with 1,3-diaminepropane and 1,4-diaminebutane as carrier ligands. It was proposed that the circumvention of cisplatin resistance was related to the steric hindrance of the diamine chelate ring and the thereby increased level of DNA interstrand crosslinks[112]. Consequently, modification of the carrier ligand results in the formation of structurally different platinum-DNA adducts and influences thereby the anticancer properties[105].

Another important feature, which is determined by both leaving group(s) and carrier ligand(s) is lipophilicity[113]. Among other factors, lipophilicity has been shown to affect absorption, transmembrane transport, bioavailability, cellular drug accumulation, lipophilic drug-receptor interactions, metabolism, pharmacological activity, as well as the toxicity of molecules. Lipophilicity of a drug has been related to its ability to cross cell membranes by means of passive diffusion and reflects the relative solubility of the drug in lipid-like (e.g., lipid bilayers of a cell membrane) and aqueous (the fluid in and out of the cells) environments.

With respect to platinum compounds, it has been suggested that increasing lipophilicity may help to overcome accumulation defects and consequently to enhance cytotoxicity in resistant cell lines[114]. Moreover, lipophilicity was shown to correlate with cytotoxic activity[115-117]. In other studies a strong relationship between intracellular platinum concentrations and lipophilicity of platinum complexes was demonstrated[114,118-120]. Most of the reported investigations have been carried out with platinum(IV) complexes or with a panel of structurally very different platinum

complexes. In order to draw convincing conclusions regarding the influence of lipophilicity on cellular accumulation, other factors like oxidation state or reactivity should be comparable between the complexes.

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2 Aims of the investigation

Oxaliplatin is a widely used third-generation anticancer drug with a better safety profile and a lack of cross-resistance to cisplatin, especially in colorectal cancer[7,13]. Nevertheless, tumor cells can develop resistance to oxaliplatin as well[57]. In contrast to the mechanisms of cisplatin resistance, which have been extensively studied, the mechanisms of resistance to oxaliplatin are less well established. This applies also for the reduced influx as a frequently documented resistance mechanism of tumor cells to platinum complexes.[57,121]. To further elucidate the contribution of reduced influx to oxaliplatin resistance, it is necessary to understand the underlying mechanisms of oxaliplatin influx.

One aim of this study was to explore general influx mechanism(s) of oxaliplatin and structurally related compounds by investigation of the influence of lipophilicity and reactivity on influx. Furthermore, it was planned to reveal, to which extent lipophilicity and reactivity influence cytotoxicity of platinum compounds and whether it is possible to overcome reduced influx in resistant cells by alteration of these physicochemical properties. For that purpose, two panels of oxaliplatin derivates were used: one panel of derivates with different amine ligands and another panel with different leaving groups. The experiments were carried out in the ileocecal colorectal adenocarcinoma cell line HCT-8 and its oxaliplatin-resistant variant HCT-8ox.

The following experiments were conducted for this purpose:

- Determination of lipophilicity of the platinum compounds
- Determination of reactivity of the platinum compounds towards nucleotides
- Determination of influx, efflux and cytotoxicity of the platinum compounds in HCT-8 and HCT-8ox cells
- Determination of resistance of HCT-8ox cells to the platinum compounds
- Determination of DNA platination and repair of platinum-DNA adducts

Another aim was to investigate the transporter-mediated influx of oxaliplatin in more detail, particularly the contribution of the transport proteins hCTR1 and hOCT1-3.

Therefore, the following additional experiments were performed:

- Determination of influx of oxaliplatin after preincubation and coincubation with different substrates and/or inhibitors of the transport proteins
- Determination of cytotoxicity of oxaliplatin after preincubation and coincubation with different substrates and/or inhibitors of the transport proteins
- Determination of gene expression levels of the transport proteins with and without oxaliplatin treatment
- Determination of subcellular localization of the transport proteins with and without oxaliplatin treatment

3 Materials and methods

3.1 Material

3.1.1 Chemicals and reagents

ALEXA Fluor™ 488-conjugated chicken anti-goat antibody	Invitrogen, Karlsruhe
2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris-HCl)	Sigma-Aldrich, Steinheim
Argon 4.6	Air Product, Hattingen
Atropine sulfate salt monohydrate	Fagron, Barsbüttel
BCA™ Albumin Standard Ampules (containig 2 mg/mL bovine serum albumin)	Pierce, Bonn
BCA™ Reagent A (containing bicinchoninic acid)	Pierce, Bonn
BCA™ Reagent B (containing 4 % cupric sulfate)	Pierce, Bonn
Boric acid	Fluka Chemie, Neu-Ulm
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim
Buffer AE (elution buffer)	Qiagen, Hilden
Buffer AL (lysis buffer)	Qiagen, Hilden
Buffer AW 1, concentrate (wash buffer)	Qiagen, Hilden
Buffer AW 2, concentrate (wash buffer)	Qiagen, Hilden
Buffer HS, concentrate (wash buffer)	Bio-Budget Technologies, Krefeld
Buffer LS, concentrate (wash buffer)	Bio-Budget Technologies, Krefeld
Buffer RL (lysis buffer)	Bio-Budget Technologies, Krefeld
CASYton, isotonic diluting solution	Schärfe System, Reutlingen
Cimetidine	Sigma-Aldrich, Steinheim
Copper sulfate · 5 H_2O	Sigma-Aldrich, Steinheim
2'-Deoxyadenosine 5'-monophosphate sodium salt (dAMP)	Sigma-Aldrich, Steinheim
2'-Deoxyguanosine 5'-monophosphate sodium salt (dGMP)	Sigma-Aldrich, Steinheim
Dietyhl pyrocarbonate (DEPC)	Sigma-Aldrich, Steinheim

4´,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich, Steinheim
Dimethylsulfoxide (DMSO)	Riedel-de Haën, Seelze
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)	AppliChem, Darmstadt
Disodium hydrogen phosphate · 2 H_2O	Merck, Darmstadt
Ethanol 96-100 % (V/V)	Merck, Darmstadt
2,2',2'',2'''-(Ethane-1,2-diyldinitrilo) tetraacetic acid disodium salt (EDTA, dinatriumsalz)	Sigma-Aldrich GmbH, Steinheim
1-Ethyl-2-[(1-ethyl-2(1H)-quinolinylidene) methyl]quinolinium iodide (decynium-22)	Sigma-Aldrich, Steinheim
Fetal calf serum (FCS)	Sigma-Aldrich, Steinheim
Fluoromount™ aqueous mounting medium	Sigma-Aldrich, Steinheim
Formaldehyde 37 % (m/V)	Riedel de Haën AG, Seelze
Goat antibodies to hCTR1, hOCT1, hOCT2 and hOCT3 [200 µg/mL]	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Helium	Praxair, Oevel
Hydrochloric acid [0.1 M and 1.0 M]	Riedel de Haën AG, Seelze
Hydrochloric acid 37 % (m/V)	Merck, Darmstadt
Isopropanol 100 %	Merck, Darmstadt
L-Glutamin solution [200 mM]	Sigma-Aldrich, Steinheim
Methanol	Merck, Darmstadt
Monosodium phosphate	Fluka Chemie, Neu-Ulm
Nitric acid 65 % (V/V), suprapur	Merck, Darmstadt
1-Octanol	Sigma-Aldrich, Steinheim
Penicillin streptomycin solution [10,000 I.E./mL, 10 mg/mL]	Sigma-Aldrich, Steinheim
Potassium chloride	Merck, Darmstadt
Potassium dihydrogen phosphate	Fluka Chemie GmbH, Neu-Ulm
Propidium iodide solution [1.5 mM]	Invitrogen, Karlsruhe
Ribonuclease A (RNase A)	Sigma-Aldrich, Steinheim
RPMI-1640 medium	Sigma-Aldrich, Steinheim
Sodium chloride	Fluka Chemie, Neu-Ulm
Sodium dihydrogen phosphate · H_2O	

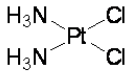
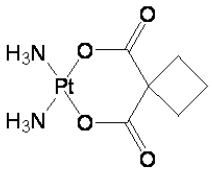
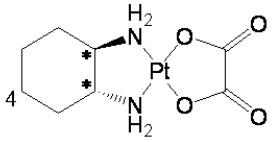
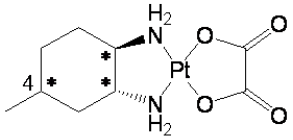
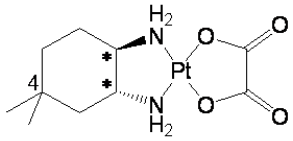
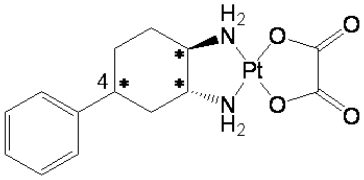
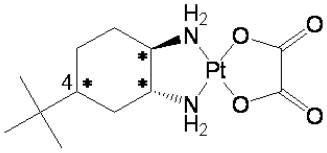
Sodium hydroxide [0.1 M and 1.0 M]	Riedel de Haën AG, Seelze
Tetraethylammonium chloride (TEA)	Sigma-Aldrich, Steinheim
Thymidine 5'-monophosphate disodium salt (TMP)	Sigma-Aldrich, Steinheim
Triton X-100	Sigma-Aldrich, Steinheim
Trypsin-EDTA solution [0.5 g porcine trypsin and 0.2 g EDTA in 100 ml]	Sigma-Aldrich, Steinheim
Ultrapure water	Obtained by Purlab Plus™ system

Platinum complexes

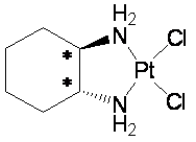
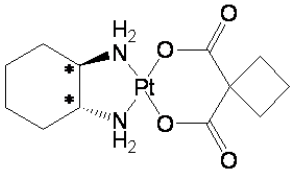
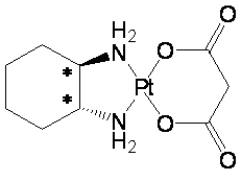
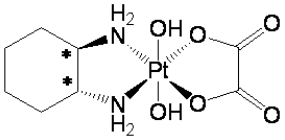
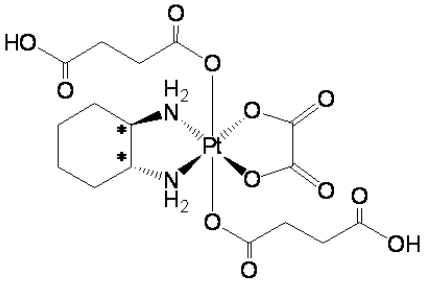
Cisplatin	Sigma-Aldrich, Steinheim
Carboplatin	Sigma-Aldrich, Steinheim
Oxaliplatin	Sigma-Aldrich, Steinheim
Oxaliplatin analogues	Synthesis of the oxaliplatin analogues was carried out by the working group of Prof. Dr. Bernhard K. Keppler according to the procedures previously published[122-128].

An overview of the structures is presented in Tab. 3-1.

*Tab. 3-1: Structure, stereochemistry and lipophilicity of the investigated platinum complexes (n.a., not applicable; *, chiral centre).*

	Complex	Stereo-chemistry at C(4)	Structure
Cisplatin	*cis*-diammine dichloroplatinum(II)	n.a.	
Carboplatin	*cis*-diammine (1,1-cyclobutanedicarboxylato) platinum(II)	n.a.	
Oxaliplatin	(*trans*-R,R-cyclohexane-1,2-diamine) oxalatoplatinum(II)	n.a.	
4	(SP-4-3)-(4-methyl-*trans*-cyclohexane-1,2-diamine) oxalatoplatinum(II)	equatorial	
5	(SP-4-3)-(4,4-dimethyl-*trans*-cyclohexane-1,2-diamine) oxalatoplatinum(II)	n.a.	
6	(SP-4-3)-(4-phenyl-*trans*-cyclohexane-1,2-diamine) oxalatoplatinum(II)	91 % axial	
7	(SP-4-3)-(4-tert-butyl-*trans*-cyclohexane-1,2-diamine) oxalatoplatinum(II)	equatorial	

*(Cont.) Structure, stereochemistry and lipophilicity of the investigated platinum complexes (n.a., not applicable; *, chiral centre).*

	Complex	Stereo-chemistry at C(4)	Structure
8	(*trans*-R,R-cyclohexane-1,2-diamine) dichloroplatinum(II)	n.a.	
9	(*trans*-R,R-cyclohexane-1,2-diamine) cyclobutane-1,1-dicarboxylatoplatinum(II)	n.a.	
10	(*trans*-R,R-cyclohexane-1,2-diamine) malonatoplatinum(II)	n.a.	
11	(*trans*-R,R-cyclohexane-1,2-diamine) dihydroxooxalatoplatinum(IV)	n.a.	
12	(*trans*-R,R-cyclohexane-1,2-diamine) bis(3-carboxypropanoato) oxalatoplatinum(IV)	n.a.	

3.1.2 Buffers and solutions

Capillary electrophoresis

Borate buffer [0.4 M, pH 9.1]

Boric acid	2.473 g
Ultrapure water	ad 100.0 mL
(adjusted to pH 9.1 with 1 M sodium hydroxide)	

dAMP stock solution [3 mM]

dAMP	12.88 mg
Ultrapure water	ad 10.0 mL

dGMP stock solution [3 mM]

dGMP	13.09 mg
Ultrapure water	ad 10.0 mL

Phosphate buffer [40 mM, pH 7.4]

Sodium dihydrogen phosphate · H_2O	552 mg
Ultrapure water	ad 100.0 mL
(adjusted to pH 7.4 with 1 M sodium hydroxide)	

Sodium chloride buffer [40 mM, pH 7.4]

Sodium chloride	234 mg
Ultrapure water	ad 100.0 mL
(adjusted to pH 7.4 with 1 M sodium hydroxide)	

TMP stock solution [3 mM]

TMP	11.88 mg
Ultrapure water	ad 10.0 mL

Platinum analysis

Cisplatin stock solution [1 mg/mL platinum]

Cisplatin	15.4 mg
Ultrapure water	ad 10.0 mL

Cisplatin working solution 1 [10 µg/mL platinum]

Cisplatin stock solution	0.1 mL
Ultrapure water	ad 10.0 mL

Cisplatin working solution 2 [1 µg/mL platinum]

Cisplatin working solution 1	1.0 mL
Ultrapure water	ad 10.0 mL

MTT assay

MTT solution

MTT	500.0 mg
Phosphate buffered saline	ad 100.0 mL

Incubation experiments

Atropine sulfate [160 mM]

Atropine sulfate salt monohydrate	44.47 mg
Ultrapure water	ad 0.4 mL

Carboplatin [800 µM]

Carboplatin	2.97 mg
Ultrapure water	ad 10.0 mL

Cell culture medium

RPMI-1640 medium	500.0 mL
Fetal calf serum	50.0 mL
Penicillin streptomycin solution	5.0 mL

Cimetidine [15 mM]

Cimetidine	3.75 mg
Ultrapure water	ad 1.0 mL

Cisplatin [800 µM]

Cisplatin	2.40 mg
Ultrapure water	ad 10.0 mL

Complex 4 [800 µM]

Complex 4	3.29 mg
Ultrapure water	ad 10.0 mL

Complex 5 [800 µM]

Complex 5	3.40 mg
Ultrapure water	ad 10.0 mL

Complex 6 [800 µM]

Complex 6	3.79 mg
Ultrapure water	ad 10.0 mL

Complex 7 [800 µM]

Complex 7	3.63 mg
Ultrapure water	ad 10.0 mL

Complex 8 [800 µM]

Complex 8	3.04 mg
Ultrapure water	ad 10.0 mL

Complex 9 [800 µM]

Complex 9	3.68 mg
Ultrapure water	ad 10.0 mL

Complex 10 [800 µM]

Complex 10	3.29 mg
Ultrapure water	ad 10.0 mL

Complex 11 [800 µM]

Complex 11	3.45 mg
Ultrapure water	ad 10.0 mL

Complex 12 [800 µM]

Complex 12	5.05 mg
Ultrapure water	ad 10.0 mL

Copper sulfate solution [500 µM]

Copper sulfate · 5 H_2O	124.8 mg
Ultrapure water	ad 1.0 mL

Decynium-22 stock solution [12 mM]

Decynium-22	54.5 mg
Dimethylsulfoxide	ad 10.0 mL

Decynium-22 working solution [500 µM]

Decynium-22 stock solution	41.7 µL
Ultrapure water	ad 1 mL

Oxaliplatin solution [12 mM]

Oxaliplatin	47.7 mg
Ultrapure water	ad 10.0 mL

Oxaliplatin solution [800 µM]

Oxaliplatin	3.18 mg
Ultrapure water	ad 10.0 mL

Phosphate buffered saline (PBS)

Sodium chloride	8.0 g
Disodium hydrogen phosphate · 2 H_2O	1.4 g
Potassium dihydrogen phosphate	0.2 g
Potassium chloride	0.2 g
Ultrapure water	ad 1000.0 mL

TEA solution [300 mM]

TEA	49.71 mg
Ultrapure water	ad 1.0 mL

Isolation of DNA

Buffer AE (elution buffer) [pH 9.0]

Tris-HCl	10.0 mM
EDTA	0.5 mM

Buffer AW 1 (wash buffer)

AW 1 concentrate*	95.0 mL
Ethanol 96 % (V/V)	ad 220.0 mL

* The manufacturer does not provide information about the exact composition.

Buffer AW 2 (wash buffer)

AW 2 concentrate*	66.0 mL
Ethanol 96 % (V/V)	ad 226.0 mL

* The manufacturer does not provide information about the exact composition.

Isolation of RNA

Buffer HS (wash buffer)

HS concentrate*	15.0 mL
Ethanol 96 % (V/V)	ad 30.0 mL

* The manufacturer does not provide information about the exact composition.

Buffer LS (wash buffer)

LS concentrate*	8.0 mL
Ethanol 96 % (V/V)	ad 40.0 mL

* The manufacturer does not provide information about the exact composition.

DEPC water [0.1 %]

DEPC	1.0 g
Ultrapure water	ad 1.0 L

Immunohistochemical staining

ALEXA Fluor™ 488-conjugated chicken anti-goat antibody solution

ALEXA Fluor™ 488-conjugated chicken anti-goat antibody	10.0 µL
Ethanol 96 % (V/V)	ad 1.0 mL

BSA solution [1 %]

BSA	0.5 g
Phospate buffered saline	ad 50.0 mL

DAPI stock solution [1 mg/mL]

DAPI	1.0 mg
Methanol	ad 1.0 mL

DAPI working solution [5 µg/mL]

DAPI stock solution	5.0 µL
Ultrapure water	ad 1.0 mL

Ethanol [90 %]

Ethanol 96-100 %	9.0 mL
Ultrapure water	ad 10.0 mL

Ethanol [70 %]

Ethanol 96-100 %	7.0 mL
Ultrapure water	ad 10.0 mL

Formaldehyde [3.7 %]

Formaldehyde 37 %	5.0 mL
Phosphate buffered saline	ad 50.0 mL

Goat antibody solution (hCTR1, hOCT1, hOCT2 or hOCT3)

Goat antibody	100 µL
BSA solution 1 %	ad 1.0 mL

Propidium iodide solution [5 µM]

Propidium iodide	3 µL
Phosphate buffered saline	ad 0.9 mL

RNase A stock solution [1 mg/mL]

RNase A	1 mg
Phosphate buffered saline	ad 1.0 mL

RNase A working solution [100 µg/mL]

RNase A stock solution	100 µL
Phosphate buffered saline	ad 1.0 mL

Triton® X-100 [0.5 %]

Triton® X-100	234 µL
Phosphate buffered saline	ad 50.0 mL

3.1.3 Cell lines

The human ileocecal colorectal adenocarcinoma cell lines HCT-8 and HCT-8ox (kindly provided by Dr. R.A. Hilger, University of Essen, Germany) were used in the study. The resistant variant (HCT-8ox) was obtained by incubation with stepwise increasing oxaliplatin concentrations. In detail, oxaliplatin was added to HCT-8 cells to a final concentration equal to the EC_{50} for 24 h. Afterwards, the cells were incubated in drug-free cell culture medium. At the point that treated cells grew as fast as untreated cells, a higher concentration of oxaliplatin was added to the cells. However, the concentration of oxaliplatin did not exceed 3- to 4-fold of the EC_{50}.

3.1.4 Consumption materials

BCA™ Protein Assay Kit	Pierce, Bonn
BIO-CERT® pipette tips	Brand GmbH & Co., Wertheim
Capillaries, uncoated (fused silica, 75 µm ID, 365 µm OD)	Beckman Coulter, Fullerton, USA
Cellstar® Cell Culture Flasks 175 cm^2	Greiner Labortechnik, Frickenhausen
Cellstar® Cell Culture Flasks 75 cm^2	Greiner Labortechnik, Frickenhausen
Cellstar® Cell Culture Flasks 25 cm^2	Greiner Labortechnik, Frickenhausen
Cellstar® conical centrifuge tubes (15 mL)	Greiner Labortechnik, Frickenhausen
Cellstar® conical centrifuge tubes (50 mL)	Greiner Labortechnik, Frickenhausen
Cellulose acetate membrane filters (0.22 µm)	Macherey-Nagel, Düren
Cover slips	Carl Roth GmbH & Co., Karlsruhe
Cryovials	Sarstedt, Nümbrecht
Disposable syringe (10 mL)	B. Braun Melsungen AG, Melsungen

Graphite tubes	Varian, Darmstadt
Microscope slides	Carl Roth GmbH & Co., Karlsruhe
MycoAlert® Mycoplasma Detection Kit	Lonza Rockland, Rockland, USA
my-Budget RNA Mini Kit	Bio-Budget Technologies, Krefeld
Pasteur pipettes	Brand GmbH & Co., Wertheim
Petri dishes	Greiner Labortechnik, Frickenhausen
Pipette tips	Brand GmbH & Co., Wertheim
Pipettes of glass	Labomedic GmbH, Bonn
Platinum hollow cathode lamps (UltrAA® lamps)	Varian, Darmstadt
QIAamp® DNA Mini Kit	Qiagen, Hilden
Reaction tubes (0.5, 1.5, 2 mL)	Greiner Labortechnik, Frickenhausen
Sample vials (2 mL, conical)	Varian, Darmstadt
Tissue culture plates, 96 wells	Greiner Labortechnik, Frickenhausen
Tissue culture plates, 6 wells	Greiner Labortechnik, Frickenhausen

3.1.5 Equipment

Axiovert® 25 inverted microscope	Carl Zeiss, Oberkochen
CASY®1 cell counter	Schärfe System, Reutlingen
CO_2 incubator	Thermo Scientific, Waltham, USA
GENESYS 6 UV/VIS spectrophotometer	Thermo Scientific, Waltham, USA
Hellma® absorption cuvettes (Suprasil® quartz)	Hellma, Mülheim
Incubator shaker Unimax® 1010	Heidolph, Schwabach
inoLab® pH level 2 pH Meter	WTW, Weilheim
Kern 770 analytical balance	Gottlieb Kern & Sohn, Albstadt
Laminar flow workstation	Heraeus, Hanau
Magnetic stirrer RMH71	Gerhardt GmbH & Co. KG, Königswinter
Microfuge® Lite centrifuge	Beckman Coulter, Brea, USA
MIKRO 200R microliter centrifuge	Hettich, Beverly, USA
Multiskan Ascent microplate photometer	Thermo Scientific, Waltham, USA
Purelab Plus water purification system	ELGA Labwater, Celle

Transferpette® -12 electronic, multi-chanel pipettes (variable volume, 0.5 - 300 µL)	BRAND, Wertheim
Transferpette® S, single-channel pipettes (variable volume, 0.5 - 1000 µL)	BRAND, Wertheim
Ultrasonic water bath	Bandelin, Berlin
Universal 32R centrifuge	Hettich, Beverly, USA
Vortexer	Gesellschaft für Laborbedarf, Würzburg
Water bath Büchi 461	Büchi, Switzerland

Atomic absorption spectrometry

Spectrometer SpectrAA® Zeeman 220	Varian, Darmstadt
Graphite tube atomizer GTA 100	Varian, Darmstadt
Programmable sample dispenser PSD 100	Varian, Darmstadt
Software SpectrAA® 220, version 2.20	Varian, Darmstadt

Capillary electrophoresis

P/ACE® 5510	Beckman Coulter, Fullerton, USA
UV detector	Beckman Coulter, Fullerton, USA

Immunohistochemical staining

Leica TCS SP2 confocal system	Leica Microsystems, Wetzlar
Zeiss ApoTome Axiovert 200M system	Carl Zeiss, Oberkochen

3.1.6 Software

Adobe® Photoshop® 6.0	Adobe Systems, San Jose, USA
GraphPad Prism™	GraphPad Software, San Diego, USA
MVA 2.0	NOVIA, Frankfurt am Main
Software P/ACE® Station, version 1.21	Beckman Coulter, Fullerton, USA
SPSS® Statistics 17	SPSS Inc., Chicago, USA
WinNonlin® Professional 5.0.1	Pharsight Corporation, Mountain View, USA

3.2 Determination of log P

Lipophilicity of the platinum complexes was quantified as log P (logarithm of 1-octanol/water partition coefficient). The log P values were determined according to the OECD guideline using the shake-flask method[129]. Weighted amounts of platinum complexes were partitioned between an aqueous and 1-octanol phase. Platinum concentrations in the aqueous phase were measured by flameless atomic absorption spectrometry. Based on these results, the partition coefficients were calculated.

3.3 Assessment of reactivity towards nucleotides

For the investigation of reactivity of platinum complexes towards the nucleotides dAMP and dGMP, a previously described method for capillary electrophoresis was modified and validated[130,131]. Briefly, 150 µM (platinum complexes with different amine ligands) or 300 µM (platinum complexes with different leaving groups) of each platinum complex were incubated with 150 µM dGMP and 150 µM dAMP under physiological conditions (4 mM NaH_2PO_4, 4 mM NaCl, pH 7.4, 37 °C) up to 72 h. A platinum-nucleotide ratio of 1:1 was chosen in case of the assessment of reactivity of platinum complexes with different leaving groups in order to estimate the rate constants of the formation of the monofunctional platinum-nucleotide adducts. The samples were collected after 0, 12, 24, 48 and 72 h of incubation (n = 3). To enhance the precision of nucleotide analysis, 4 µL internal standard (TMP, 3 mM in buffer containing 4 mM NaH_2PO_4, 4 mM NaCl, pH 7.4) were added to 100 µL sample. The measurement was carried out on a P/ACE™ 5510 instrument controlled by the P/ACE™ station software. An uncoated capillary (75 µm ID, effective separation length 57 cm) was assembled in the P/ACE™ cartridge. The capillary was thermostated at 19 °C. Injections were performed at 5 psi for 5 s. A voltage of 20 kV was applied for all experiments. Detection was performed using UV absorption at 254 nm. Prior to each analysis the capillary was flushed with running buffer (400 mM borate buffer, pH 9.1) for 1 min. After each analysis the capillary was rinsed with sodium hydroxide (0.1 M) for 1 min, followed by ultrapure water for 1 min.

3.4 Determination of the rate constants

The rate constants for the reaction between the platinum complexes ([Pt(DACH)Y_2], Y_2 being the leaving group) and the nucleotides, dGMP and dAMP, were calculated based on a second-order reaction. To simplify the calculations, it was focused exclusively on the formation of the monofunctional platinum-nucleotide adducts. Consequently, for the rates (ν_1 and ν_2) and the rate constants (k_1 and k_2) the following relationship holds (Eqs. 1 and 2):

$$v_1 = -\frac{d[dGMP]}{dt} = k_1 \bullet [Pt(DACH)Y_2] \bullet [dGMP] \quad (1)$$

$$v_2 = -\frac{d[dAMP]}{dt} = k_2 \bullet [Pt(DACH)Y_2] \bullet [dAMP] \quad (2)$$

Under the condition that at any time of the reaction

$$[Pt(DACH)Y_2] = [dGMP] + [dAMP] \quad (3)$$

the rate constants k_1 and k_2 were estimated by non-linear regression of the nucleotide concentration-time data using WinNonlin® Professional 5.0.1.

3.5 Platinum analysis

The platinum concentrations were measured by flameless atomic absorption spectrometry using a modification of the procedure described by Kloft et al., which was validated by Dr. Anne C. Pieck[132,133]. Because the validation was carried out on the same instrument used in this study a revalidation was not necessary. In brief, an atomic absorption spectrometer equipped with a graphite tube atomizer, a programmable sample dispenser and a platinum hollow cathode lamp were used. The temperature program included an ashing step at 1,300 °C and an atomization step at 2,700 °C. The lower limit of quantification (LLOQ) was 1 ng/mL.

Samples with platinum concentrations below the LLOQ of the flameless atomic absorption spectrometry were analyzed using a validated adsorptive stripping voltammetry method[134]. With this method the detection limit could be reduced to 0.4 pg/mL. In brief, after drying the samples were mineralized by a high-pressure

asher (HPA, Kürner, Rosenheim, Germany)[135]. Platinum was then quantified by adsorptive stripping voltammetry using a Metrohm Polarecord 693 VA Processor with 694 VA Stand (Metrohm, Herisau, Switzerland). The standard addition technique was used to determine the platinum concentrations[134]. The measurements were performed in the working group of Dr. Günther Weber.

In the course of time, a faster method of determination of platinum in samples below the LLOQ of flameless atomic absorption spectrometry was available: the inductively coupled plasma mass-spectrometry (ICP-MS)[136]. Samples were lyzed with an aqueous solution containing 1 % nitric acid for 24 h on a water bath at 78 °C. For the quantification of platinum a Varian 820 ICP-MS detector was used and volumes of 1.0 mL were injected. Accuracy and precision of the method were determined during the analysis. Each ICP-MS analysis resulted from five replicate measurements consisting of 20 scans of the isotops of interest. Quality control samples were used to insure the accuracy of the measurements. The measurement of the samples was carried out in the working group of Dr. Ralf A. Hilger.

3.6 NMR spectroscopy

Oxaliplatin (10 mM) and cimetidine (150 mM) were incubated in PBS/DMF mixture (1:1) at 37 °C. After 1 h, 2 h, 3 h and 16 h, ^{195}Pt NMR spectra of the reaction mixture were recorded on a Bruker DPX 300 spectrometer with a 5 mm multinucleus probe. The spectra were calibrated with respect to external K_2PtCl_4 at δ = - 1,614 ppm. The working group of Prof. Dr. Jan Reedijk carried out the measurements.

3.7 Cell culture

Cells were grown in RPMI-1640 medium after adding 10 % fetal calf serum, 100 I.E./mL penicillin and 0.1 mg/mL streptomycin and cultivated as monolayers at 37 °C in a humidified atmosphere containing 5 % CO_2. Cells were routinely checked for mycoplasma contamination by DAPI staining and using MycoAlert® Mycoplasma Detection Kit. Backups of all cells were frozen with 10 % DMSO and stored in liquid

nitrogen. Every 12 passages, a new backup of cells was thawed to ensure that the resistance status was unchanged during long-time cultivation.

The advantages of this cell model were the high sensitivity of HCT-8 cells to oxaliplatin and the high level of resistance of HCT-8ox cells to oxaliplatin. The HCT-8ox cell line acquired resistance after oxaliplatin treatment bearing the advantage of developing oxaliplatin-specific characteristics.

Cell count was carried out using a Casy™1 cell counter, which works via electronic pulse area analysis. Beside the number of cells, cell volume, aggregation and viability were determined.

3.8 Cytotoxicity assay

Cytotoxicity of the platinum complexes was assessed using an MTT-based assay[137]. Briefly, cells were plated in 96-well microtiter plates (approximately 5,000 cells/well) and pre-incubated with growth medium for up to six hours. Then medium was removed. Stock solutions of platinum complexes in ultrapure water (0.8 mM) were diluted in medium and nine subsequent dilutions were added to the cells in triplicate (100 μL/well). In some experiments cells were pre- or coincubated with copper sulfate (coincubation: 50 and 100 μM), TEA (coincubation: 10 μM, 1 and 10 mM), atropine sulfate (Fig. 3-1A, preincubation: 1 h, 1 mM; coincubation: 0.1 and 1 mM), cimetidine (Fig. 3-1B, preincubation: 6 h, 1.5 mM; coincubation: 0.1 mM or 1 mM) or decynium-22 (Fig. 3-1C, coincubation: 0.1 and 1 μM).

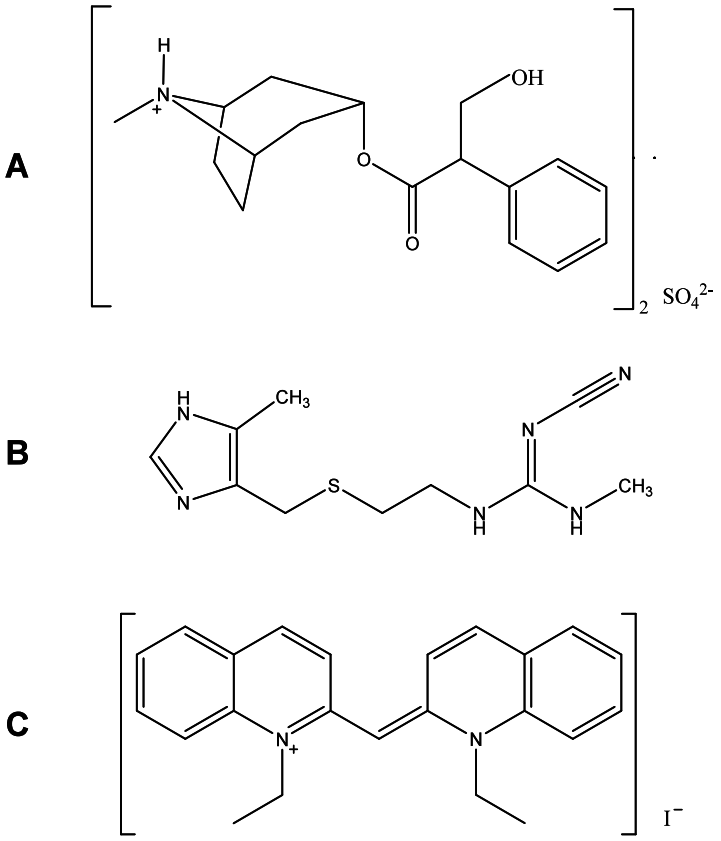


*Fig. 3-1 Structure of **A** atropine sulfate, **B** cimetidine and **C** decynium-22.*

After 72 h of incubation, 20 µL of a 5 mg/mL MTT solution was added to each well, and the cells were incubated at 37 °C for about 1 h. Subsequently, medium was discarded and 100 µL DMSO was added to dissolve the formazan crystals. Absorbance of the converted dye was measured at 570 nm with background subtraction at 690 nm using a microplate photometer. The results were analyzed and the pEC_{50} values (pEC_{50} = -log EC_{50}, EC_{50} is the drug concentration that produces 50 % of the maximum possible response) were estimated with the GraphPad Prism™ analysis software package using non-linear regression (sigmoidal dose response, variable slope). The resistance factor (RF) was calculated by dividing the EC_{50} in the resistant variant by the EC_{50} in the respective sensitive cell line.

3.9 Measurement of intracellular platinum concentration

3.9.1 Influx of oxaliplatin analogues

In order to characterize the influx of the oxaliplatin analogues, 1 x 10^6 cells per well were seeded in six-well plates. After attachment (5 – 6 h), cells were incubated with 100 µM platinum complex up to 2 h. After certain time points the medium was discarded quickly and cells were washed with 1 mL ice-cold PBS (phosphate buffered saline, pH adjusted to 7.4). Then cells were trypsinized, resuspended in fresh drug-free medium and centrifuged for 1 min at 4 °C and 1,520 g. The supernatant was discarded and the pellet was washed twice in 1 mL ice-cold PBS. After centrifugation for 1 min at 18,620 g the supernatant was discarded again and the cell pellet was frozen at -20 °C until further analysis. Immediately after thawing the cells were lyzed with concentrated nitric acid for 1 h on a water bath at 80 °C. Then intracellular platinum concentrations were measured by flameless atomic absorption spectrometry or adsorptive stripping voltammetry. Platinum concentrations were calculated in relation to the mean cell volume (as measured with Casy™1 cell counter).

3.9.2 Influx of oxaliplatin

In order to characterize the influx of oxaliplatin, three different types of influx experiments were conducted. In each experiment 1 x 10^6 cells per well were seeded in six-well plates. After the attachment (5 – 6 h), cells were incubated with oxaliplatin under different conditions.

In the first set of experiments cells were treated with different concentrations of oxaliplatin (0.02, 0.1, 0.5, 1.0, 1.5 mM) over a period of 2 h. In the second set of experiments cells were incubated with 100 µM oxaliplatin up to 2 h. In the third set of experiments cells were pre- or coincubated with substrates and/or inhibitors of transport proteins. In general, preincubation experiments were only performed if an effect was found in coincubation experiments. Cells were treated with copper sulfate (substrate of hCTR1; coincubation: 50 and 100 µM), TEA (substrate of hOCT1-3; coincubation: 10 µM, 1 mM and 10 mM), atropine sulfate (Fig. 3-1A, inhibitor of

hOCT1; preincubation: 1 h, 1 mM; coincubation: 1 mM), cimetidine (Fig. 3-1B, inhibitor of hOCT2; preincubation: 1 h, 1.5 mM, coincubation: 0.15 and 1.5 mM) or decynium-22 (Fig. 3-1C, inhibitor of hOCT3; coincubation: 0.1 and 1 µM).[60,77,78,138] In each experiment, cells were incubated with oxaliplatin for 2 h. Afterwards, cells were collected as described above (see 3.9.1).

3.9.3 Long-term influx of oxaliplatin in presence of cimetidine

To characterize the influence of cimetidine on the influx of oxaliplatin in more detail, long-term experiments were performed. In each experiment 5×10^5 cells per well were seeded in six-well plates. After the attachment (5 – 6 h), cells were preincubated with 1.5 mM cimetidine for 12 h and subsequently incubated with 20 µM oxaliplatin up to 24 h or cells were coincubated with 1.5 mM cimetidine and 20 µM oxaliplatin up to 24 h. After 2, 4, 6, 8, 10, 12 and 24 h of oxaliplatin treatment cells were collected as described above (see 3.9.1). In contrast to the above-described collection of the cells, after the first centrifugation step at 1,520 g and the subsequent resuspension of the cell pellet in ice-cold PBS an aliquot of 20 µL was removed for protein determination (see 3.10.3). Both, cell pellet and aliquot, were frozen at -20 °C until further analysis.

3.9.4 Efflux

In order to characterize the efflux of platinum, 1×10^6 cells per well were seeded in six-well plates and allowed to attach for 5 to 6 h. Afterwards they were incubated with 100 µM platinum complex for 2 h. One part of the cells was collected as described above. The other part of the cells was washed two times with 1 mL PBS and incubated for 2, 5, 10, 60 and 120 min with drug-free medium. Subsequently, the cells were collected as described above (see 3.9.1).

3.10 Protein determination

In some influx experiments time after plating cells was too long to act on the assumption that the number of cells remained unchanged. The doubling time of both

cell lines determined prior to the experiments was about 14 h. Therefore, in all experiments, in which cells grew longer than 14 h, the intracellular platinum concentration was calculated in ng/mg protein.

The protein concentration was determined using the bicinchoninic assay (BCA™ Protein Assay Kit) according to the manufacturers instructions. A validation with respect to the calibration curve (linearity and working range, precision and accuracy, lower limit of quantification) was already conduced in our working group[131]. Therefore, the validation in this study was restricted to the examination of the conditions of the sample preparation. The most important step during the sample preparation is the solubilization of the cell suspension. To get reliable results it is important that the cells are completely solubilized.

3.10.1 Standards and quality control samples

Six non-zero standard concentrations were prepared out of the albumin standard (SL) ampule provided by the manufacturer (Tab. 3-2). Ultrapure water was used as diluent. Standards and quality control samples were prepared out of different charges of stock solution.

Tab. 3-2 Preparation of standards and quality control samples.

	Volume of diluent [µL]	Volume and source of BSA [µL]	Final BSA concentration [µg/mL]
Standards			
SL1	--	--	2,000
S1	1,600	400 of SL	400
S2	250	750 of S1	300
S3	500	500 of S1	200
S4	600	300 of S2	100
S5	625	375 of S3	75
S6	300	300 of S4	50
Quality control samples			
SL2	--	--	2,000
QC1	825	175 of SL2	350
QC2	875	125 of SL2	250
QC3	925	75 of SL2	150

3.10.2 Experimental procedure

Protein samples (20 µL) were thawed. Standards and quality control samples were prepared and 40 µL of each concentration was pipetted into a vial. To assure that the experimental conditions were the same for samples, standards and quality control samples, all solutions were treated in the same way. 10 µL of sodium hydroxide (1 M) was added to each sample and 20 µL to each standard and quality control. All solutions were sonicated for 30 min at room temperature. Afterwards, the solutions were neutralized by addition of the same volume of hydrochloric acid (1 M) as before of sodium hydroxide. Dilutions with ultrapure water were performed where necessary in order to bring the protein concentration of the samples within the calibration range.

20 µL of each solution was transferred to a 96-well microtiter plate. Standards were determined in triplicate, quality control samples and samples in duplicate. The

subsequent procedure was carried out as described by the manufacturer (microplate procedure).

If the following conditions were met, the experiment was accepted[139]:

- The deviation of standards was ≤ 15 % and the deviation of the LLOQ ≤ 20 % from nominal concentration.
- At least four out of six standards met the above criteria, including the LLOQ and the calibration standard at the highest concentration.
- The coefficient of correlation was ≥ 0.99 (weighted linear regression, 1/x).
- At least 67 % (two out of three) of the quality control samples were within ± 15 % of their respective nominal values.

3.10.3 Partial validation of the sample preparation

Aliquots for the determination of proteins were removed during the collection of cells after long-term incubation with oxaliplatin and/or cimetidine (see 3.9.3). The time after seeding cells varied in this experiment between 7 and 48 h. Consequently, the concentration of cells in the aliquots differed within a wide range. Therefore, cell lysis became a crucial factor in the determination of the concentration of proteins. To be sure that even in samples with a high concentration of cells all cells were lyzed, the method of sample preparation was validated. Under optimal cell lysis conditions one would expect a linear relationship between protein and cell concentration.

Suspension of HCT-8 and HCT-8ox cells with 1×10^6, 2×10^6, 3×10^6, 4×10^6 cells/mL were prepared. Aliquots of 20 µL were removed and frozen at -20 °C for further analysis. At six different days six aliquots of each cell concentration were thawed and the protein concentration was determined as described above. Finally, the protein concentration was related to the cell concentration.

3.11 Measurement of platinum-DNA adduct formation and repair

For the determination of the platinum-DNA adduct formation 5×10^6 cells were incubated with 100 µM platinum complex up to 4 h and subsequently washed with

1 mL ice-cold PBS. Afterwards, cells were trypsinized, resuspended in fresh drug-free medium and centrifuged for 1 min at 4 °C and 1,520 g. The supernatant was discarded and the pellet was washed twice in 1 mL ice-cold PBS. After centrifugation for 1 min at 18,620 g the supernatant was discarded again. The cell pellet was frozen at -20 °C until further analysis.

Repair experiments were performed by incubating 1×10^6 cells for 4 h with oxaliplatin. HCT-8 cells were incubated with 100 µM oxaliplatin and HCT-8ox cells with 200 µM oxaliplatin. The different concentrations were chosen to achieve a comparable extent of platinum-DNA adducts in each cell line at the beginning of the experiment. Subsequent to the incubation with oxaliplatin, cells were washed once in 1 mL ice-cold PBS and afterwards, drug-free cell culture medium was added to each well. After 0, 2, 4 or 6 h cells were collected as described above.

DNA platination was determined using a previously published method adapted to tumor cells[140]: Subsequent to the isolation with solid-phase extraction (QIAmp® DNA Mini Kit) DNA concentrations were measured by UV photometry and platinum concentrations by flameless atomic absorption spectrometry, adsorptive stripping voltammetry or ICP-MS, as appropriate. Based on these results the platinum-nucleotide ratio [platinum atoms/10^6 nucleotides] was calculated as a measure of the extent of DNA platination. The platinum-nucleotide ratio reflects the net rate of adduct formation and repair.

DNA quantification and determination of platinum in adduct samples using flameless atomic absorption spectrometry was validated by Dr. A. Pieck with respect to accuracy and precision[133].

3.12 Gene expression analysis

In each experiment 1.2×10^5 cells per well were seeded in six-well plates. On the next day oxaliplatin (20 µM) was added to some wells. After another 2 h or 24 h oxaliplatin-containing medium was removed and cells were further cultured in platinum-free medium for 24 h. Cells treated for 24 h with oxaliplatin without a subsequent wash-out phase and untreated cells were maintained in parallel. The experiment, in which cells were treated for 24 h with oxaliplatin without a wash-out

phase, was included to secure that the effect of the incubation with oxaliplatin was not masked by the wash-out phase. Afterwards, treated and untreated cells were harvested by trypsinization and RNA was isolated using my-Budget RNA Mini Kit. The optical density ratio (260/280 nm) of all RNA samples was between 2.08 and 2.36. RNA integrity was confirmed by agarose gel electrophoresis (2 % in HEPES buffer containing 10 mM EDTA, 50 mM CH_3COONa x 3 H_2O, 200 mM HEPES, pH 7.2, and 1.7 % formaldehyde). 2 µg of RNA was mixed with 6x loading dye and heated to 65 °C for 5 min. Then 2 µL of SYBR® Green II (Applied Biosystems, Foster City, USA) was added and the samples were loaded onto the gel. Reverse transcription was performed with 1 µg RNA by using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) and 35 pM oligo$(dT)_{23}$ anchored primer. Reverse transcription was performed at 37 °C for 2 h. Reverse transcription mixtures were diluted with 1x Tris-EDTA buffer pH 7.4, and quantitative real-time PCR (qRT-PCR) was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA) according to the manufacturer´s instructions. Sequences of the used primers are listed in Tab. 3-3. Housekeeping genes were identified using geNorm[141,142]. Analysis of differential gene expression was carried out using the $\Delta\Delta C_t$-method[143]. After normalization to the gene of interest (GOI) the results were rescaled by setting the normalized mRNA expression of the relevant transporter in untreated HCT-8 cells to 1 (= rescaled normalized expression).

Verification of RNA integrity, qRT-PCR, identification of housekeeping genes and analysis of differential gene expression were performed in the working group of Prof. Dr. Matthias Kassack.

Tab. 3-3 Sequences of the primers used in qRT-PCR.

Gene	Accession number	Left 5´→ 3´	Right 5´→ 3´
ß-Actin (*ACTB*)	AY582799	TCCTTCCTGGGC ATGGAGT	GCACTGTGTTGG CGTACAG
Beta-2-microglobulin (*B2M*)	BC064910	CACCCCCACTGA AAAAGATG	CAAACCTCCATGA TGCTG
Glucuronidase beta (*GUS*)	NM_000181	TTCACCAGGATC CACCTCTG	AGCACTCTCGTC GGTGACTG
Hypoxanthine-phosphoribosyltrans-ferase 1 (*HPRT1*)	NM_000194	CTGGCGTCGTGA TTAGTG	CACACAGAGGGC TACAATG
60S (Human) Acidic ribosomal protein P1 (*HUPO*)	BC070194	AGCTCTGGAGAA ACTGCTG	CAGCAGCTGGCA CCTTATTG
Phospholipase A2 (*PhosA2*)	M86400	GACAGCTTTTGAT GAAGCCATTG	TCCACAATGTCAA GTTGTCTCTCAG
Ribosomal protein L13 (*RPL13*)	BC071929	GCTCATGAGGCT ACGGAAAC	TATTGGGCTCAG ACCAGGAG
Transferrin receptor (p90, CD71) (*TFR*)	BC001188	GACTTTGGATCG GTTGGTG	CAGTAACCGGAT GCTTCAC
Ubiquitin-conjugating enzyme E2D2 (*UBE2D2*)	NM_003339	ACCACCTAAGGTT GCATTTAC	TAGATCCGAGCA ATCTCAGG
Human copper transporter 1 (*hCTR1*)	BC061924	AGCTGGAGAAAT GGCTGGAG	AGGTGAGGAAAG CTCAGCATC
Human organic cation transporter 1 (*hOCT1*)	NM_003057	ACCTCGGAGTGA TGGTGTGTTC	CCAACACCGCAA ACAAAATG
Human organic cation transporter 2 (*hOCT2*)	NM_003058	CACCGAGTTTAAC CTGGTATGTG	GGCCAAACCTGT CTGCTATG
Human organic cation transporter 3 (*hOCT3*)	NM_021977	GTCAGCGAGTTT GACCTTGTC	CCATACCTGTCTG CTGCATAG

3.13 Immunohistochemical staining

Immunohistochemical staining was done one day after seeding cells on cover slips. In some experiments, cells were pre-treated with either 20 µM oxaliplatin for 2 h, sometimes followed by a subsequent wash-out phase of 2 h. After three rinses with PBS, cells were fixed with 3.7 % formaldehyde in PBS for 15 min at room temperature. After fixation, cells were washed three times with PBS and permeabilized with 0.5 % Triton X-100 in PBS for 30 min. In some cases, 0.02 % Triton X-100 was used for permeabilization in parallel experiments. No differences in the staining results depending on Triton X-100 concentration were observed. Cells were again rinsed three times with PBS and afterwards unspecific binding was blocked with 1 % BSA in PBS for 1 h. An incubation for 90 min at 37 °C with a primary antibody against the respective transport protein (hCTR1, hOCT1, hOCT2, hOCT3) was followed. Subsequently, cells were washed three times with PBS and incubated for 90 min at 37 °C with the secondary ALEXA Fluor™ 488-conjugated antibody. Antibody solutions were diluted in PBS containing 1 % BSA. To stain the nucleus with propidium iodide, cells were treated with RNase A (100 µg/mL in PBS) for 30 min at 37°C, washed with PBS and incubated with 5 µM propidium iodide in PBS for 15 min at 37 °C. After the final washing steps, cells were gradually dehydrated in an ethanol series of 70 %, 90 %, 100 %, for 1 min each. The cover slips were mounted in Fluoromount™ medium for microscopic observations. Images were recorded using a Leica TCS SP2 confocal system or a Zeiss ApoTome Axiovert 200M system for brightfield images at the Laboratory for Molecular Developmental Biology, LIMES Institute, University of Bonn. Images were normalized to the fluorescence of cells stained only with the secondary antibody. Each image is a representative of six images.

3.14 Statistical analysis

Although the results of the Kolmogorov-Smirnov test suggested a Gaussian distribution of the data from the cell culture experiments, the sample number was too small to exclude a non-Gaussian distribution. Therefore, the median was chosen as measure of central tendency. Consequently, differences were analyzed using the

non-parametric Mann-Whitney U test or the Kruskal-Wallis test, as appropriate. Correlation analyses were performed using the non-parametric Kendall tau rank correlation. In contrast, the EC_{50} values are generally accepted to be log-normally distributed. In this case, it was regarded as appropriate to calculate the mean pEC_{50} values and to compare the results by performing Student's t-test. The results of the investigation of the gene expression were first analyzed using ANOVA. When a significant difference was found, Tukey's test was used to determine which of the means differed. P values of ≤ 0.05 were considered significant.

4 Results

4.1 Oxaliplatin analogues with different amine ligands

4.1.1 Platinum complexes

The introduction of substituents at position 4 of the cyclohexane ring creates an additional chiral center. Thus, compounds **4**, **6** and **7** contain alkyl groups in equatorial and/or in axial position. The synthesized complexes **4** and **7** feature exclusively equatorial substitution, and **6** is mainly axially substituted (Tab. 3-1)[124,125,144].

4.1.2 Determination of log P

The results of the log P determination are shown in Tab. 4-1[131]. As expected lipophilicity of the compounds with different amine ligands increases in the following order: cisplatin < carboplatin < oxaliplatin < **4** < **5** < **6** < **7**.

Tab. 4-1 log P values of the investigated platinum complexes.

Compound	log P
Cisplatin	-2.53
Carboplatin	-2.30
Oxaliplatin	-1.76
4	-1.28
5	-0.68
6	-0.28
7	0.42

4.1.3 Reactivity towards nucleotides

During the incubation of the platinum complexes with nucleotides, platinum-nucleotide adducts were formed. The reactivity of the platinum complexes was determined based on the decrease in the amount of free nucleotide (Fig. 4-1 and Fig. 4-2)[131].

A faster decrease in the concentration of dGMP compared to the concentration of dAMP was observed in presence of all tested platinum complexes. Oxaliplatin and its analogues with different amine ligands showed a comparable reactivity towards the nucleotides. For comparison, the reactivity of carboplatin and cisplatin was also determined. Cisplatin turned out to be the most reactive and carboplatin the least reactive compound.

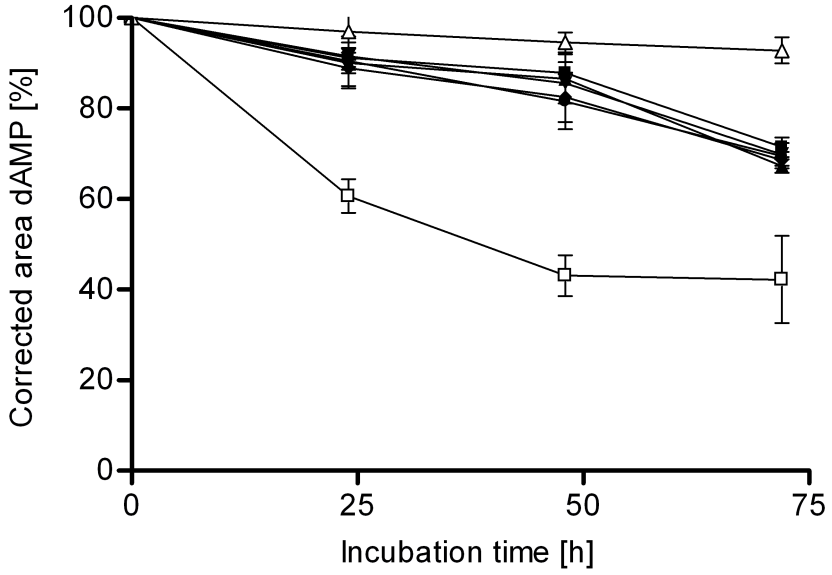


Fig. 4-1 Reduction of the corrected peak area of the nucleotide dAMP during incubation with platinum complex: cisplatin (□), carboplatin (△), oxaliplatin (♦), 4 (●), 5 (▲), 6 (▼), 7 (■) (n = 3, mean ± SD).

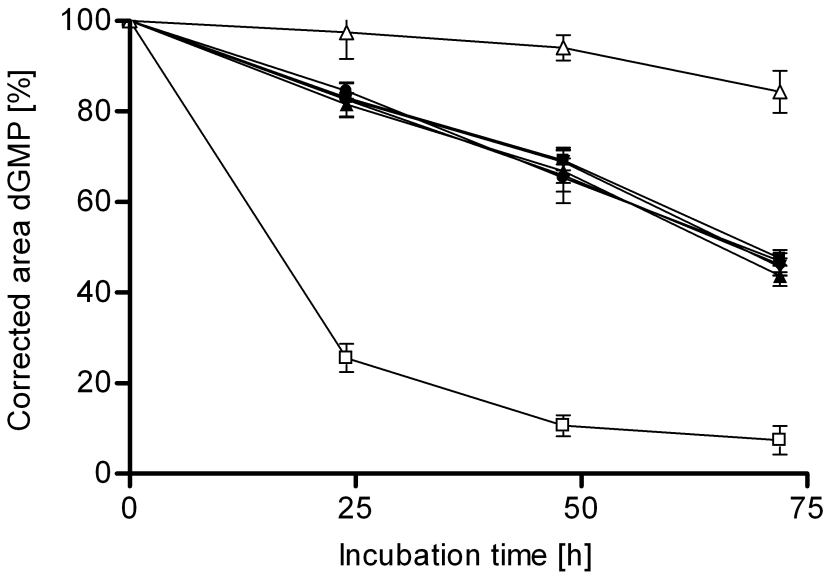


Fig. 4-2 Reduction of the corrected peak area of the nucleotide dGMP during incubation with platinum complex: cisplatin (□), carboplatin (△), oxaliplatin (♦), 4 (●), 5 (▲), 6 (▼), 7 (■) (n = 3, mean ± SD).

4.1.4 Platinum accumulation

4.1.4.1 Influx

During the incubation experiments the platinum accumulation within the cells was measured. The platinum accumulation reflects the net rate of influx and efflux. Because of the infeasibility to determine each process separately, conditions were chosen to enhance one of the processes. Thus, a high extracellular concentration of platinum complex was used to boost the influx.

During the incubation with oxaliplatin and its analogues up to 2 h platinum accumulation exhibited an approximately linear increase (except **7**) (Fig. 4-3 and Fig. 4-4).

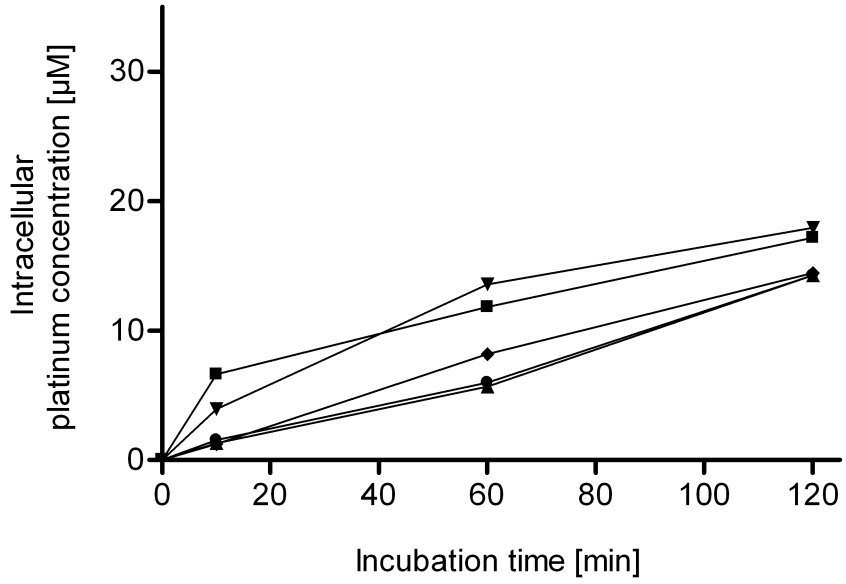


*Fig. 4-3 Intracellular platinum concentration in HCT-8 cells during incubation with 100 µM platinum complex: oxaliplatin (♦), **4** (●), **5** (▲), **6** (▼), **7** (■) (n = 6 - 18, median, for reasons of clarity a measure of variation is not shown).*

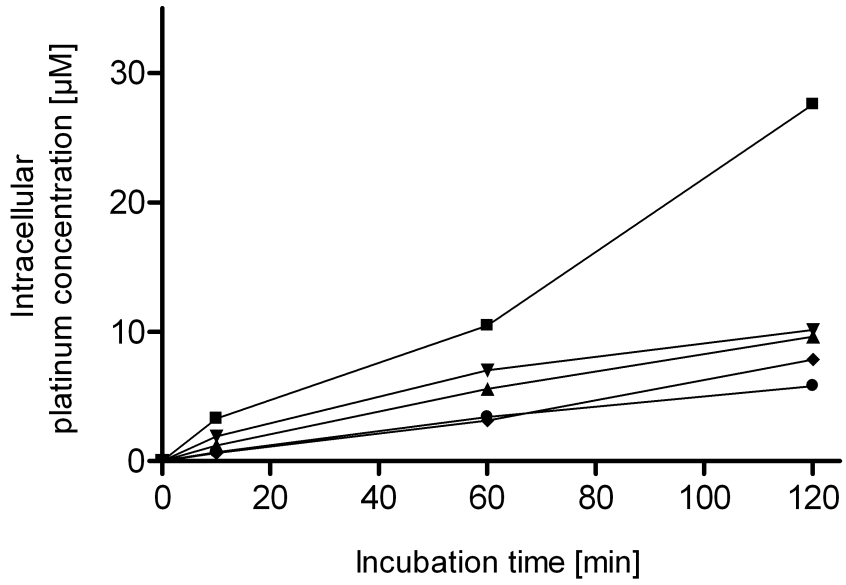


*Fig. 4-4 Intracellular platinum concentration in HCT-8ox cells during incubation with 100 µM platinum complex: oxaliplatin (♦), **4** (●), **5** (▲), **6** (▼), **7** (■) (n = 6 - 18, median; for reasons of clarity a measure of variation is not shown).*

A reduction of the platinum accumulation in the resistant cell line was found for all compounds except **7**. This compound showed a higher platinum accumulation in the resistant cell line after 2 h of incubation compared to the sensitive cell line.

For comparison of the concentration-time profiles of the different platinum compounds, it was differentiated between early and late influx. The influx rate within 10 min of incubation was used as an indicator for early influx (early influx rate). Due to the approximately linear increase of the platinum accumulation between the 10th and 120th min observed in both cell line pairs a linear regression of the median concentration-time profile was performed for each complex. The slope obtained reflected the influx rate in the late influx phase (late influx rate) and was used as an indicator for late influx.

The relationship between lipophilicity and early influx rate is shown in Fig. 4-5.

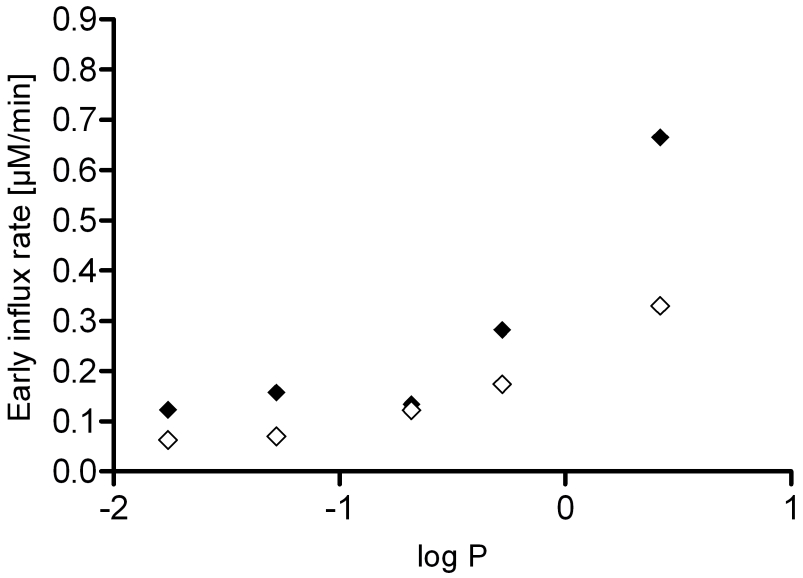


*Fig. 4-5 Dependence of the early influx rate in HCT-8 (♦) and HCT-8ox (◊) cells after incubation with 100 µM platinum complex for 10 min on lipophilicity of oxaliplatin and platinum complexes **4 - 7** (n = 6 - 18, median; for reasons of clarity a measure of variation is not shown).*

Already after 10 min of incubation a reduced influx rate of some complexes in the resistant cell lines was observed. The difference was statistically significant for

oxaliplatin, **4** and **7**. Moreover, good to relatively strong correlations were found in both cell lines ($0.80 \leq r \leq 1.00$). These findings suggest an influence of lipophilicity on the early influx of the platinum complexes.

In Fig. 4-6 the calculated late influx rates of the complexes are displayed.

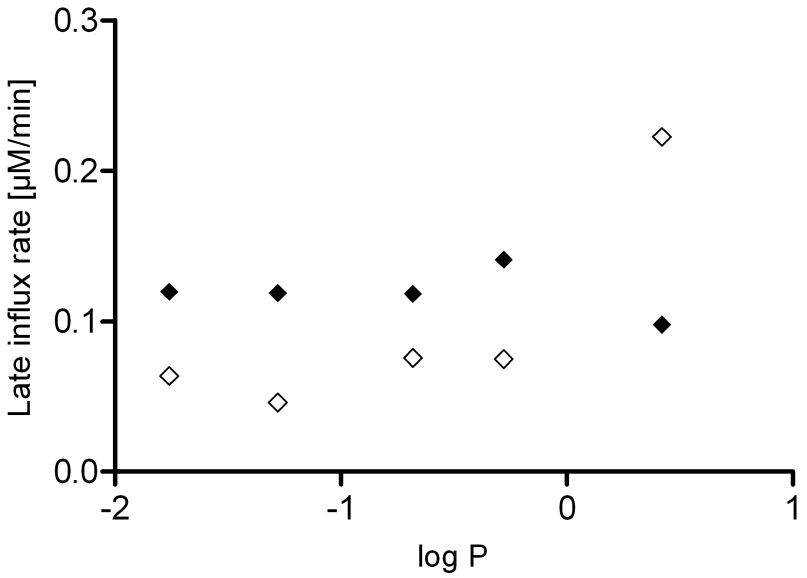


*Fig. 4-6 Dependence of the late influx rate in HCT-8 (♦) and HCT-8ox (◊) cells after incubation with 100 µM platinum complex on lipophilicity of oxaliplatin and the platinum complexes **4 - 7** (n = 2 - 6, median; for reasons of clarity a measure of variation is not shown).*

Late influx rates of a given compound in resistant cells were slower compared to the sensitive cell line. The only exception was **7** exhibiting a higher late influx rate in the resistant cell line. No significant correlation between lipophilicity and late influx rates was found in the investigated cell lines.

4.1.4.2 Efflux

Investigation of efflux was important for two reasons. Firstly, the reduced platinum accumulation in the resistant cell lines may be a result of increased efflux. Secondly, efflux may contribute to the striking differences in platinum accumulation after incubation with **7** as compared to oxaliplatin (Fig. 4-3 and Fig. 4-4).

Therefore, efflux rate of oxaliplatin as the least lipophilic compound and **7** as the most lipophilic compound was assessed by measuring the platinum accumulation after incubation with the complexes and subsequent incubation in drug-free medium (Fig. 4-7 and Fig. 4-8).

In the ileocecal colorectal adenocarcinoma cell line pair oxaliplatin efflux was slow, only after 1 h the efflux rate seemed to be enhanced. The efflux of **7** was fast only in the first minutes. Neither the efflux of oxaliplatin nor the efflux of **7** differed noteworthy between HCT-8 and HCT-8ox cells.

These results suggest that differences in efflux of oxaliplatin and **7** between sensitive and resistant cells are negligible. Therefore, the different cellular accumulation of both compounds in the resistant cell line seems to be due to differences in influx rather than efflux.

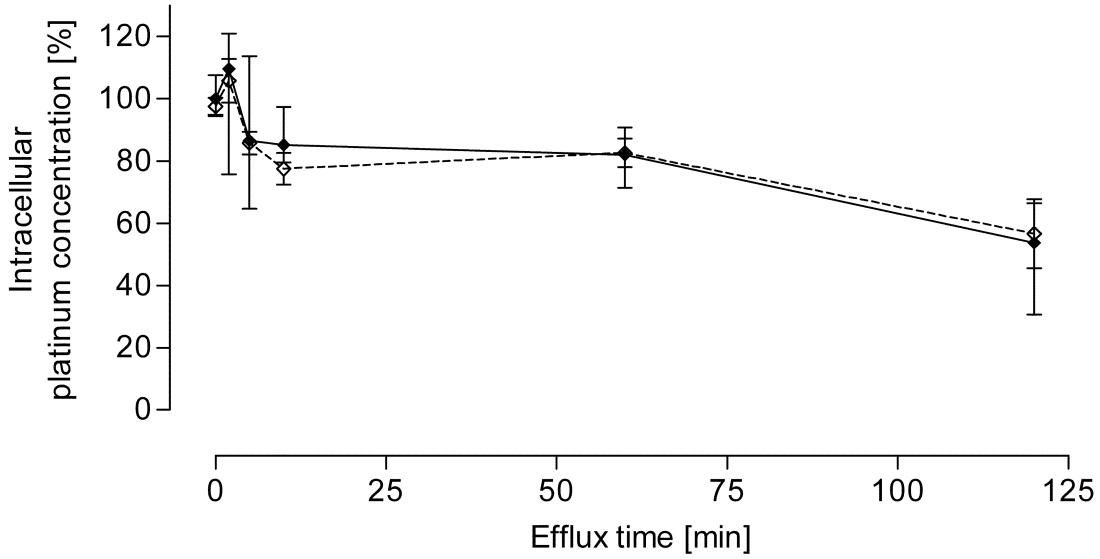


Fig. 4-7 Intracellular platinum concentration in HCT-8 (♦) and HCT-8ox (◊) cells after 2 h of incubation with oxaliplatin and subsequent incubation with drug-free medium (n = 6, median ± IQR). The median platinum concentration at the end of the 2 h incubation period was set to 100 %.

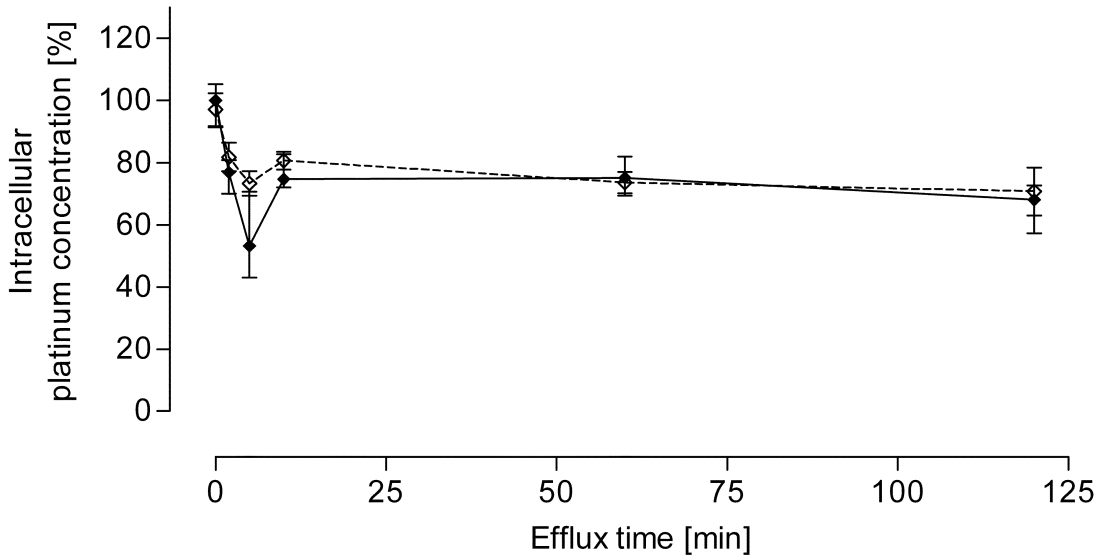


*Fig. 4-8 Intracellular platinum concentration in HCT-8 (♦) and HCT-8ox (◊) cells after 2 h of incubation with complex **7** and subsequent incubation with drug-free medium (n = 6, median ± IQR). The median platinum concentration at the end of the 2 h incubation period was set to 100 %.*

4.1.5 Cytotoxicity

The sensitivity of each cell line to oxaliplatin and its analogues is presented in Tab. 4-2.

*Tab. 4-2 pEC_{50} values and resistance factors (RF) of oxaliplatin and the platinum complexes **4 - 7** in the HCT-8 cell line pair (n = 3, mean ± SE).*

Compound	**pEC_{50}** (EC_{50})		RF
	HCT-8	**HCT-8ox**	
Oxaliplatin	6.11 ± 0.03 (0.8 µM)	5.11 ± 0.03 (7.8 µM)	10.0
4	5.31 ± 0.09 (4.9 µM)	4.45 ± 0.01 (35.5 µM)	7.3
5	5.19 ± 0.05 (6.5 µM)	4.58 ± 0.06 (26.3 µM)	4.0
6	4.51 ± 0.06 (30.9 µM)	4.16 ± 0.01 (69.2 µM)	2.2
7	4.14 ± 0.05 (72.4 µM)	4.10 ± 0.05 (79.4 µM)	1.1

The low cytotoxicity of the most lipophilic compound **7** compared to the other compounds is remarkable. Furthermore, HCT-8ox cells did not show any resistance against **7**.

The determined pEC_{50} values were inversely correlated with the lipophilicity of the complexes. A strong correlation was found in both investigated cell lines The correlation was found to be at least good for both investigated cell lines ($-0.80 \leq r \leq -1.00$). Increased lipophilicity of the oxaliplatin analogues is hence associated with lower cytotoxicity.

In order to investigate the possibility to overcome resistance using more lipophilic platinum complexes, correlation between lipophilicity and the resistance factor was also tested. A strong inverse correlation was observed in the ileocecal colorectal adenocarcinoma cell line pair ($r = -1.00$, $p < 0.01$).

4.1.6 DNA platination

In order to address the question to which extent oxaliplatin and **7** (as the least and the most lipophilic complex, respectively) form DNA adducts, the DNA platination was analyzed (Fig. 4-9).

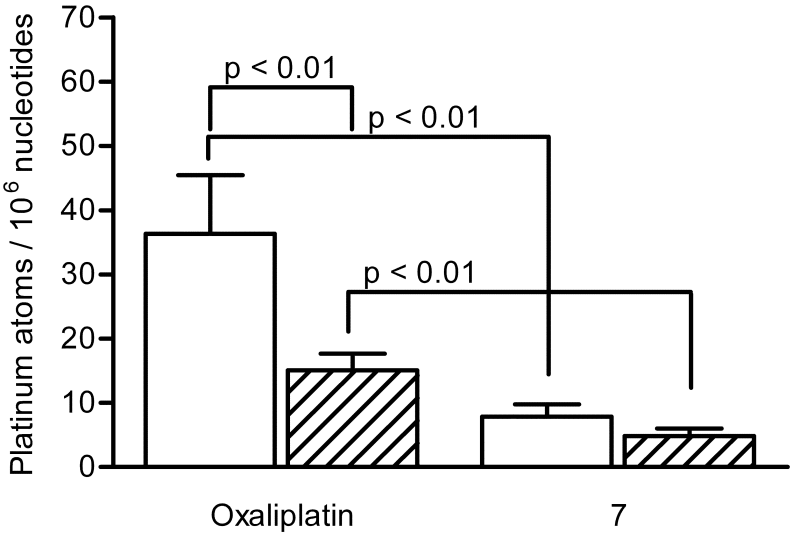


*Fig. 4-9 DNA platination in HCT-8 (unfilled column) and HCT-8ox (striped column) cells after incubation with oxaliplatin and **7** for 4 h (n = 6, median ± IQR; Mann-Whitney U test).*

A 2.4-fold higher level of oxaliplatin-DNA adducts was found in HCT-8 as compared to HCT-8ox cells, whereas the extent of DNA platination by **7** was only 1.6-fold higher and did not differ significantly between HCT-8 and HCT-8ox cells. Comparison of DNA platination in presence of oxaliplatin and **7** shows that oxaliplatin formed on average 4.6-fold more adducts in HCT-8 and 3.1-fold more adducts in HCT-8ox cells.

4.1.7 Repair of platinum-DNA adducts

The reason for the low degree of DNA platination in HCT-8ox compared to HCT-8 cells after incubation with oxaliplatin might be a result of an enhanced repair of platinum-DNA adducts in HCT-8ox cells. To figure out, if enhanced repair represents a resistance mechanism of HCT-8ox cells, the changes in the amount of platinum-DNA adducts in both cell lines after incubation with oxaliplatin and subsequent incubation in drug-free medium were investigated (Fig. 4-10).

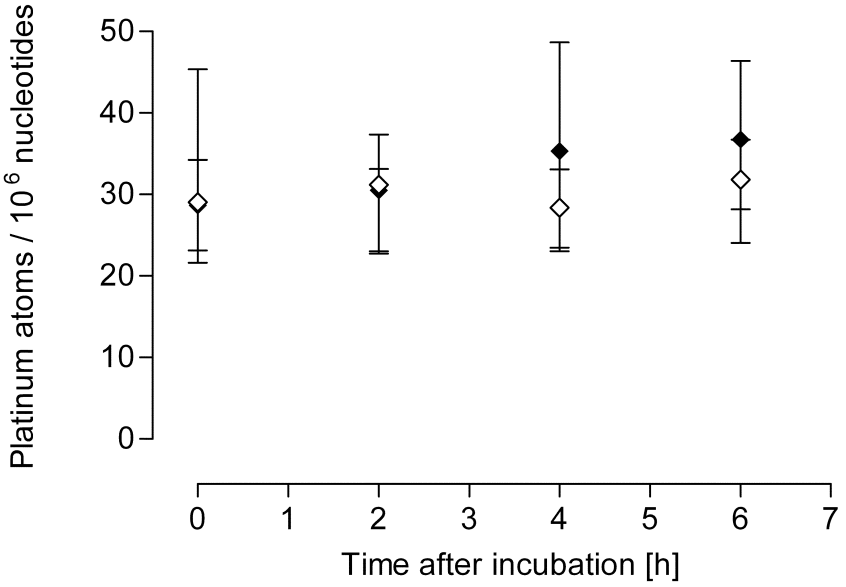


Fig. 4-10 DNA platination in HCT-8 (♦) and HCT-8ox (◊) cells after incubation with oxaliplatin for 4 h (n = 6, median ± IQR).

As shown in Fig. 4-10, the amount of platinum-DNA adducts was comparable between sensitive and resistant cells and remained nearly constant. Even after 6 h of incubation in drug-free medium, no significant changes could be observed.

4.2 Oxaliplatin analogues with different leaving groups

4.2.1 Determination of log P

The results of the log P determination are shown in Tab. 4-3. As expected lipophilicity of the compounds with different leaving groups increases in the order: **12** < **11** < oxaliplatin < **10** < **8** < **9**.

Tab. 4-3 log P values of the investigated platinum complexes.

Compound	log P
Oxaliplatin	-1.76
8	-1.30
9	-0.86
10	-1.56
11	-2.34
12	-2.67

4.2.2 Reactivity towards nucleotides

During the incubation of the platinum complexes with nucleotides, platinum-nucleotide adducts were formed. Reactivity of the platinum compounds was determined based on the decrease in the amount of free nucleotide (Fig. 4-11 and Fig. 4-12) and the respective rate constants were estimated (Tab. 4-4). For comparison, reactivity of carboplatin and cisplatin was studied as well. The order of the rate constants of the reaction between the platinum complex and dGMP (k_1) and dAMP (k_2), respectively, was cisplatin > **8** > oxaliplatin > **10** > **9** ≈ carboplatin.

In general, the rate constant k_1 was at least 2-fold higher compared to the rate constant k_2, which means that each platinum complex preferentially bound to dGMP. Reactivity of the platinum(IV) complexes was negligible.

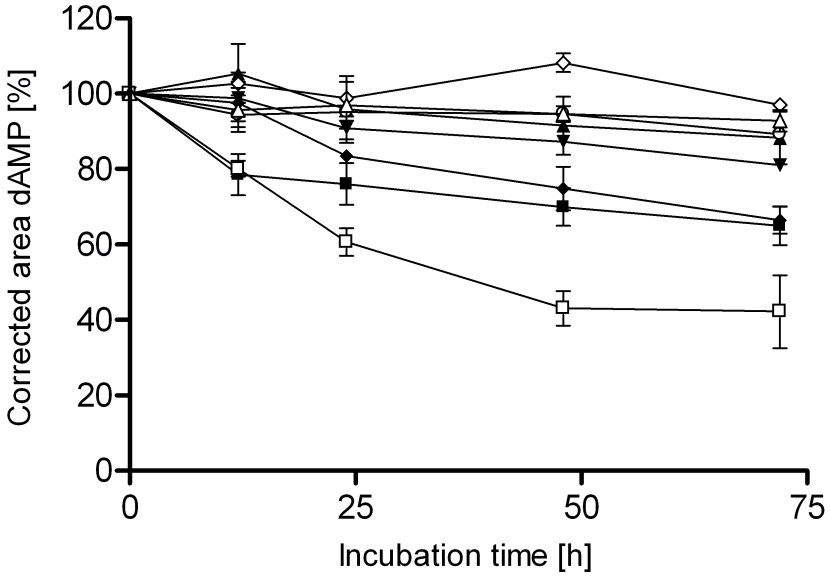


*Fig. 4-11 Reduction of the corrected peak area of the nucleotide dAMP during incubation with platinum complex: cisplatin (□), carboplatin (△), oxaliplatin (♦), **8** (■), **9** (▲), **10** (▼), **11** (◊), **12** (○) (n = 3, mean ± SD; some error bars are smaller than data points).*

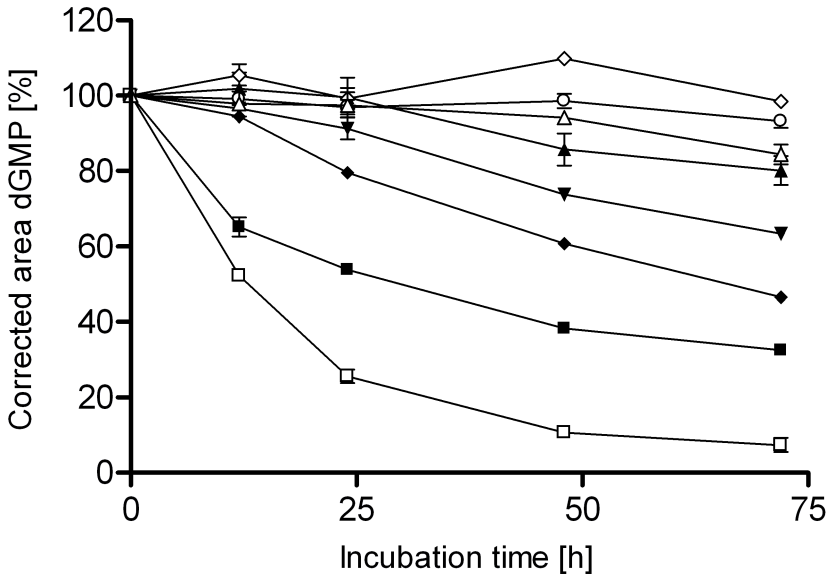


*Fig. 4-12 Reduction of the corrected peak area of the nucleotide dGMP during incubation with platinum complex: cisplatin (□), carboplatin (△), oxaliplatin (♦), **8** (■), **9** (▲), **10** (▼), **11** (◊), **12** (○) (n = 3, mean ± SD; some error bars are smaller than data points).*

Tab. 4-4 Rate constants of the reaction between platinum complexes with different leaving ligands and nucleotides dGMP (k_1) and dAMP (k_2), respectively, (n = 3, mean ± SE).

Compound	**k_1 [10^{-6}•M^{-1} • h^{-1}]**	**k_2 [10^{-6}•M^{-1} • h^{-1}]**
Cisplatin	270 ± 24.0	102 ± 10.0
Carboplatin	7 ± 1.0	3 ± 1.0
Oxaliplatin	48 ± 2.0	25 ± 2.3
8	89 ± 2.9	26 ± 2.0
9	13 ± 1.5	7 ± 1.2
10	28 ± 0.6	13 ± 0.3
11	n.a.	n.a.
12	n.a.	n.a.

4.2.3 Platinum accumulation

As already explained, the platinum accumulation was measured within cells after incubation with high extracellular concentrations of platinum compound, because under these conditions the influx was supposed to govern the measured concentration-time profiles (see 4.1.4.1).

During incubation with oxaliplatin and its analogues up to 2 h, platinum accumulation increased approximately linear in the investigated cell lines (Fig. 4-13 and Fig. 4-14). A reduction of platinum accumulation in the resistant cell line (Fig. 4-14) was found for all compounds except **9** and **11**. The platinum(IV) complexes (**11** and **12**) accumulated only to a small extent in both cell lines.

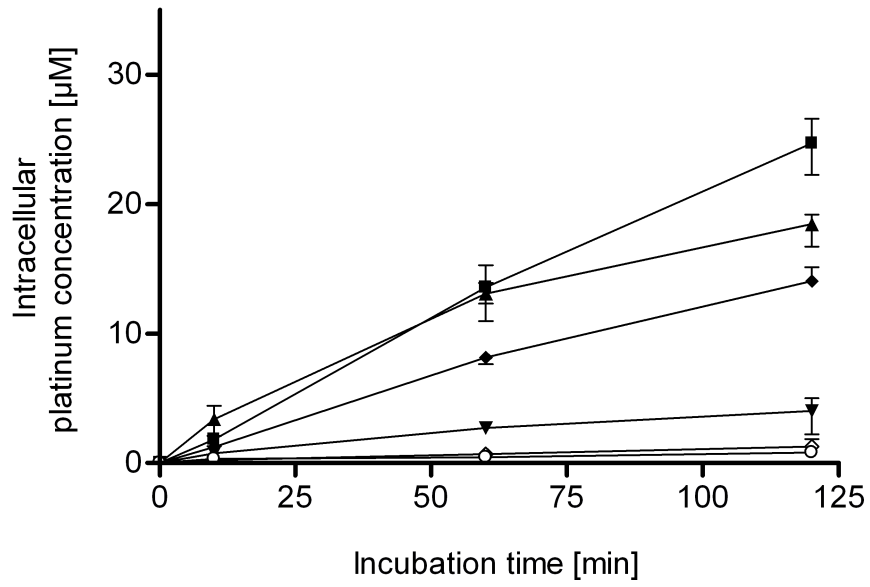


*Fig. 4-13 Intracellular platinum concentration in HCT-8 cells during incubation with 100 µM platinum complex up to 2 h: oxaliplatin (♦), **8** (■), **9** (▲), **10** (▼), **11** (◊), **12** (○) (n = 6 - 18, median ± IQR; some error bars are smaller than data points).*

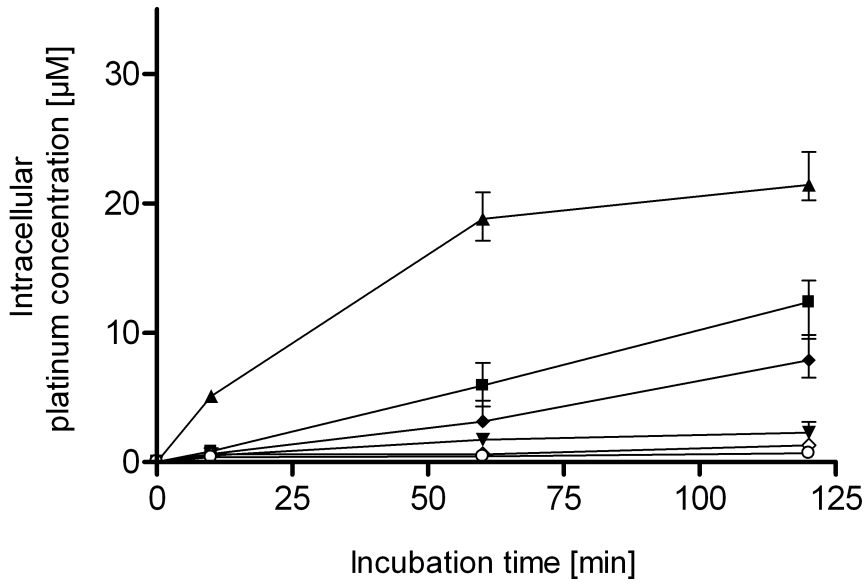


*Fig. 4-14 Intracellular platinum concentration in HCT-8ox cells during incubation with 100 µM platinum complex up to 2 h: oxaliplatin (♦), **8** (■), **9** (▲), **10** (▼), **11** (◊), **12** (○) (n = 6 - 18, median ± IQR; some error bars are smaller than data points).*

In the next step, the influence of both, lipophilicity and reactivity, on the influx rate of the oxaliplatin analogues in the early and late influx phase was investigated (Fig. 4-15, Fig. 4-16, Fig. 4-17 and Fig. 4-18).

The oxaliplatin analogues with different amine ligands (**4** - **7**) described in chapter 4.1 were included in the examination of the influence of lipophilicity, but excluded from the reactivity studies due to their similar reactivity. Firstly, the observations in the early influx phase are described in detail (Fig. 4-15 and Fig. 4-16).

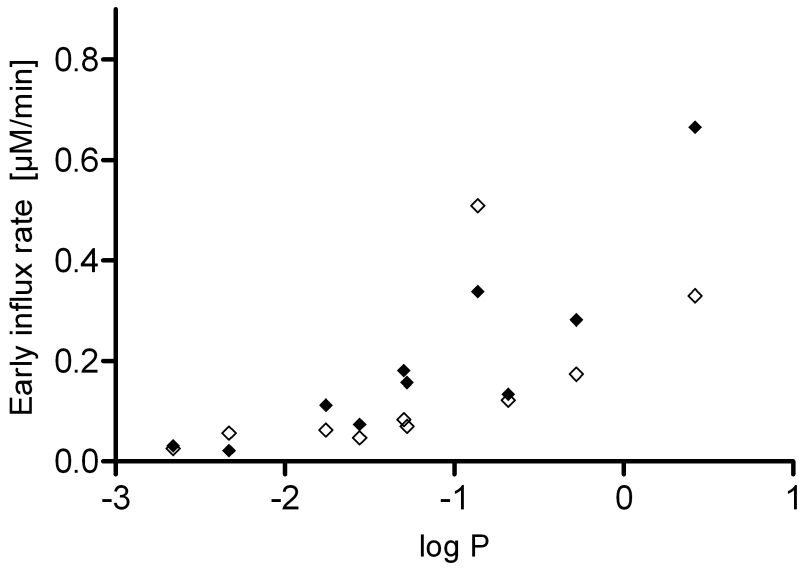


*Fig. 4-15 Dependence of the early influx rate in HCT-8 (♦) and HCT-8ox (◊) cells after incubation with 100 µM platinum complex for 10 min on lipophilicity of oxaliplatin and **4 - 12** (n = 6 - 18, median; for reasons of clarity a measure of variation is not shown).*

Already after 10 min of incubation a reduced early influx rate of some complexes in the resistant cell line was found compared to the sensitive counterpart, except **9** and **11**, where the contrary was observed. A good correlation between log P values and early influx rates was found in HCT-8 and HCT-8ox cells (HCT-8: r = 0.689, p = 0.006; HCT-8ox: r = 0.733, p = 0.003) (Fig. 4-15).

Moreover, an increase in reactivity (k_1) led to an enhanced influx rate: **10** < oxaliplatin < **8**. Despite the low reactivity of **9**, the early influx rate was high (Fig. 4-16).

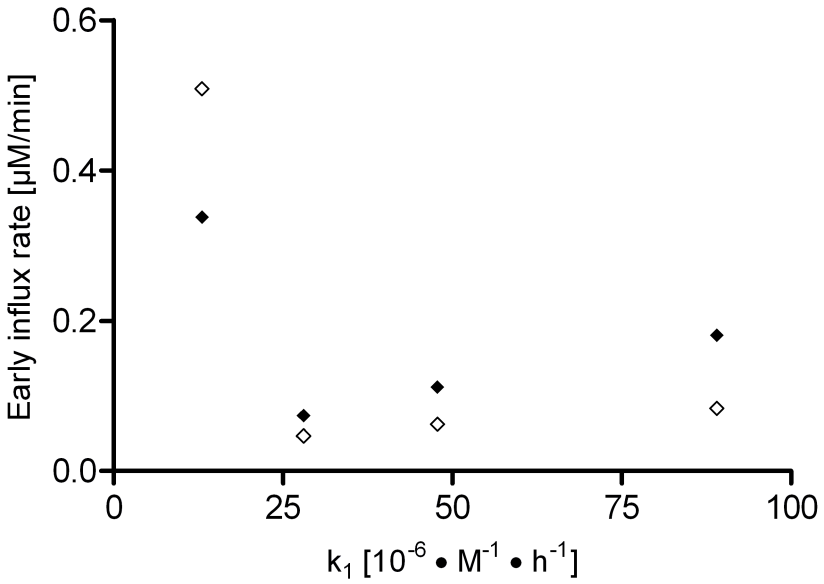


*Fig. 4-16 Dependence of the early influx rate in HCT-8 (♦) and HCT-8ox (◊) cells after incubation with 100 µM platinum complex for 10 min on reactivity towards dGMP of oxaliplatin and **8** - **10** (n = 6 - 18, median; for reasons of clarity a measure of variation is not shown).*

Secondly, the late influx rates were analyzed (Fig. 4-17 and Fig. 4-18). The comparison of a given compound in sensitive and resistant cells showed a reduction of the late influx rate in the resistant cell line, apart from **7** and **9**, which accumulated faster in HCT-8ox compared to HCT-8 cells (Fig. 4-17). Correlation between log P values and late influx rates was weak and not significant in HCT-8 cells, however, good and significant in HCT-8ox cells ($r = 0.644$, $p = 0.009$).

In both cell lines late influx rates were influenced by the reactivity of the platinum complexes (Fig. 4-18). Comparable to the early influx phase, but more pronounced, an increase in k_1 led to an increase in the late influx rate: **10** < oxaliplatin < **8.** Again the late influx rate of the least reactive complex, **9**, was high.

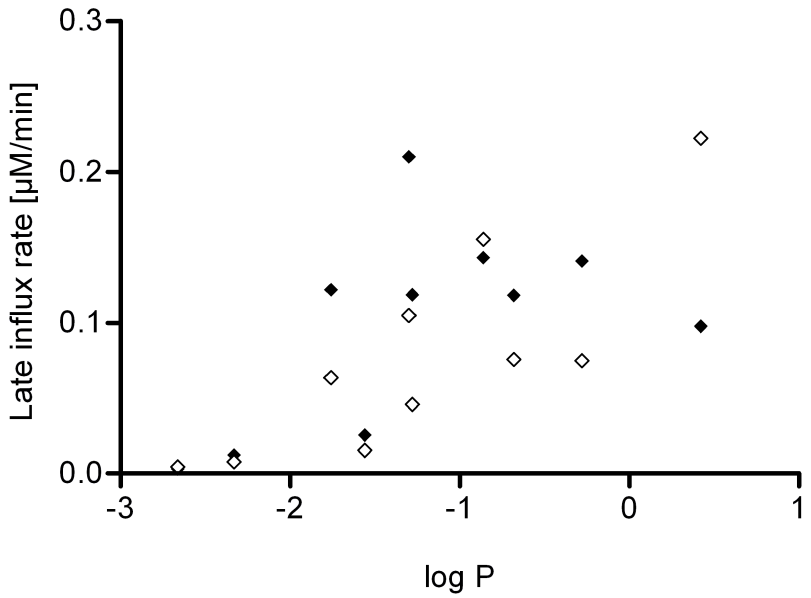


*Fig. 4-17 Dependence of the late influx rate in HCT-8 (♦) and HCT-8ox (◊) cells after incubation with 100 µM platinum complex on lipophilicity of oxaliplatin and **4** - **12** (n = 2 - 6, median; for reasons of clarity a measure of variation is not shown).*

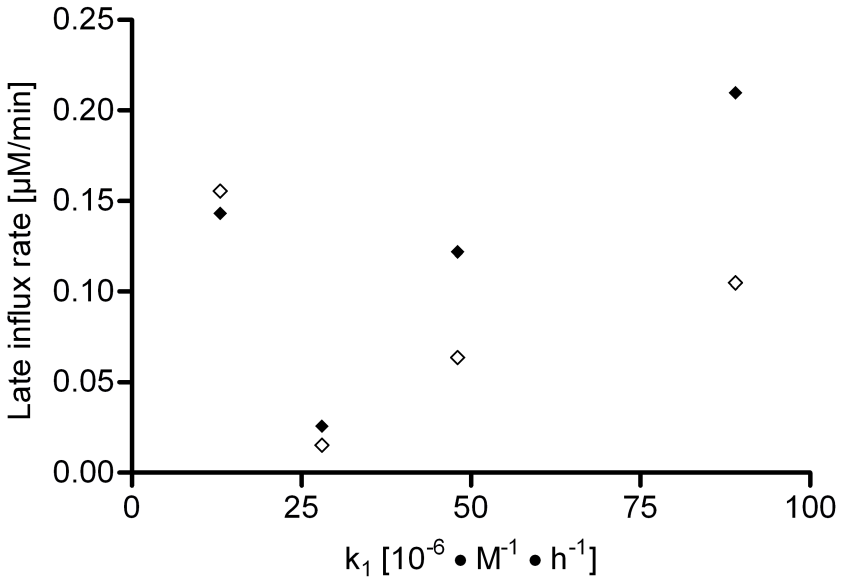


*Fig. 4-18 Dependence of the late influx rate in HCT-8 (♦) and HCT-8ox (◊) cells after incubation with 100 µM platinum complex on reactivity towards dGMP of oxaliplatin and the platinum complexes **8** - **10** (n = 2-6, median; for reasons of clarity a measure of variation is not shown).*

At last, the early and late influx rates of a given compound were compared (Tab. 4-5). The late influx rate was in general comparable or lower than the early influx rate. An exception represented **8** with a higher influx rate in the late phase than in the early phase in both cell lines.

Tab. 4-5 Early and late influx rates of the platinum compounds in HCT-8 and HCT-8ox.

Compound	HCT-8		HCT-8ox	
	Early influx rate	**Late influx rate**	**Early influx rate**	**Late influx rate**
Oxaliplatin	0.123	0.119	0.063	0.064
4	0.157	0.119	0.070	0.046
5	0.134	0.118	0.122	0.076
6	0.282	0.141	0.174	0.075
7	0.665	0.098	0.330	0.222
8	0.181	0.210	0.084	0.105
9	0.338	0.143	0.509	0.155
10	0.074	0.026	0.047	0.015
11	0.022	0.012	0.057	0.008
12	0.031	0.003	0.026	0.004

In order to investigate the influence of the amine ligands and leaving groups in more detail, additionally, the platinum accumulation upon incubation with cisplatin and carboplatin was assessed for comparison (Fig. 4-19 and Fig. 4-20).

In Fig. 4-19 the concentration-time profiles of the platinum accumulation after incubation with cisplatin and its analogue **8** are presented. Despite different amine ligands the accumulation of both complexes was not significantly different in HCT-8ox cells. In HCT-8 cells the accumulation of **8** was reduced to 70 – 80 % (10 min: $p = 0.0043$, 60 min: $p = 0.0043$, 2 h: n.s.). Interestingly, the concentration-time profiles of carboplatin and its analogue **9** were completely different, although these complexes also only differ in their amine ligands (Fig. 4-20). Carboplatin was taken up to a higher extent in sensitive cells, in contrast to **9**. The accumulation of **9**

compared to carboplatin was 3-fold higher in HCT-8 and more than 10-fold in HCT-8ox cells (10 min: p = 0.0022, 60 min: p = 0.0022, 2 h: p = 0.0022).

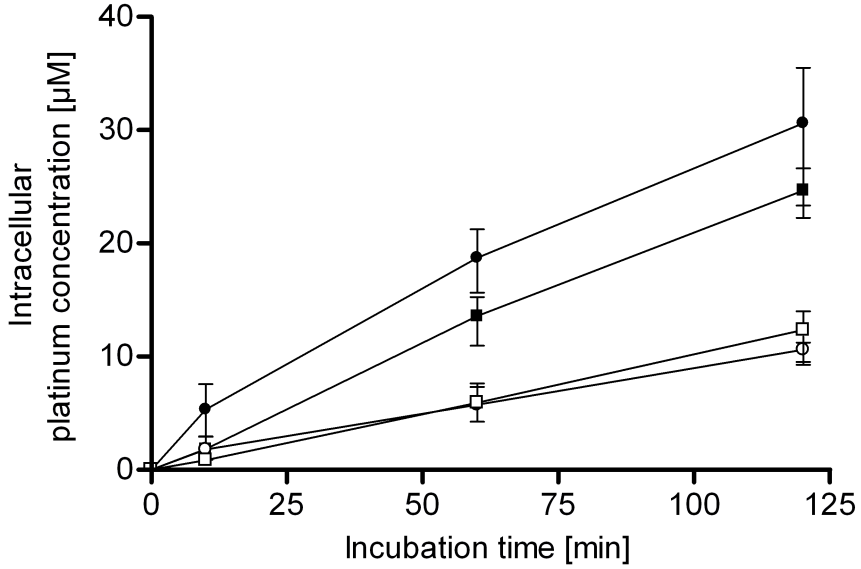


*Fig. 4-19 Intracellular platinum concentration of cisplatin (●, ○) and **8** (■, □) in HCT-8 (filled symbols) and HCT-8ox (open symbols) cells after incubation with 100 µM platinum complex up to 2 h (n = 6, median ± IQR).*

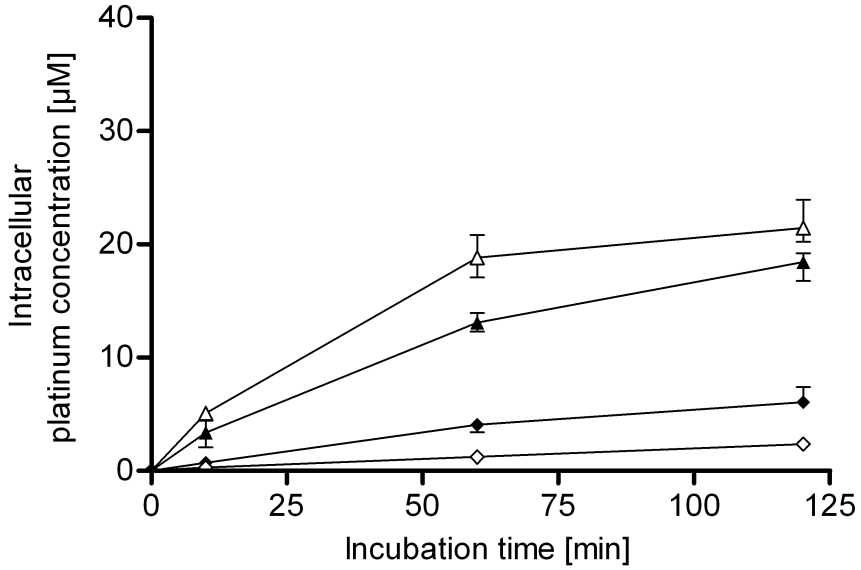


*Fig. 4-20 Intracellular platinum concentration of carboplatin (♦, ◇) and **9** (▲, △) in HCT-8 (filled symbols) and HCT-8ox (open symbols) cells after incubation with 100 µM platinum complex up to 2 h (n = 6, median ± IQR).*

4.2.4 Cytotoxicity

The cytotoxicity of all compounds was investigated in the ileocecal colorectal adenocarcinoma cell line pair and the influence of reactivity and lipophilicity on cytotoxicity was evaluated.

Both platinum(IV) complexes exhibited low cytotoxicity and low resistance factors Tab. 4-6). The resistance of HCT-8ox cells towards cisplatin and carboplatin was also low, but the cytotoxic activity of carboplatin and especially cisplatin was clearly higher than cytotoxicity of platinum(IV) complexes. **8** was the most potent platinum complex followed by oxaliplatin, **10** and **9** in both cell lines.

*Tab. 4-6 pEC_{50} values and resistance factors (RF) of cisplatin, carboplatin, oxaliplatin and platinum complexes **8** - **12** in HCT-8 and HCT-8ox cells (n = 3 - 11, mean ± SE).*

Compound	**pEC_{50}** (EC_{50})		RF
	HCT-8	**HCT-8ox**	
Cisplatin	5.15 ± 0.09 (7.1 µM)	4.69 ± 0.03 (20.4 µM)	2.9
Carboplatin	4.10 ± 0.06 (79.4 µM)	3.80 ± 0.07 (158.5 µM)	2.2
Oxaliplatin	6.11 ± 0.03 (0.8 µM)	5.11 ± 0.03 (7.8 µM)	10.0
8	6.24 ± 0.05 (0.6 µM)	5.38 ± 0.06 (4.2 µM)	7.2
9	5.60 ± 0.03 (2.5 µM)	4.78 ± 0.02 (16.6 µM)	6.6
10	5.94 ± 0.04 (1.1 µM)	5.00 ± 0.03 (10.0 µM)	8.6
11	3.84 ± 0.04 (144.5 µM)	3.36 ± 0.04 (437.0 µM)	3.0
12	3.91 ± 0.03 (123.0 µM)	3.28 ± 0.01 (525.0 µM)	4.3

The hypothesis that with increased reactivity towards nucleotides cytotoxicity of the complexes also increases, could be confirmed for the oxaliplatin analogues (HCT-8: $r = 1.000$, $p < 0.01$, HCT-8ox: $r = 1.000$, $p < 0.01$) (Fig. 4-21). However, cytotoxicity of

all investigated platinum(II) complexes (oxaliplatin and **4 - 10**) was also influenced by lipophilicity (HCT-8: r = -0.714, p = 0.013; HCT-8ox: r = -0.786, p = 0.006) (Fig. 4-22).

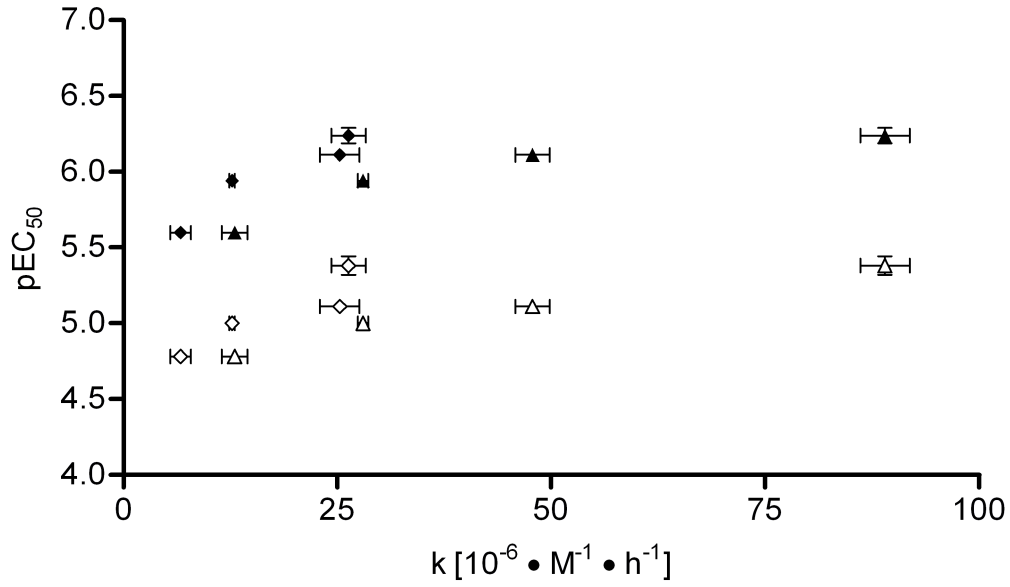


*Fig. 4-21 Relationship between reactivity (k_1: ▲, k_2: ♦, mean ± SE) and cytotoxicity of oxaliplatin and **8**, **9** and **10** (pEC_{50} values, n = 3 - 11, mean ± SE) in HCT-8 (filled symbols) and HCT-8ox (open symbols) cells.*

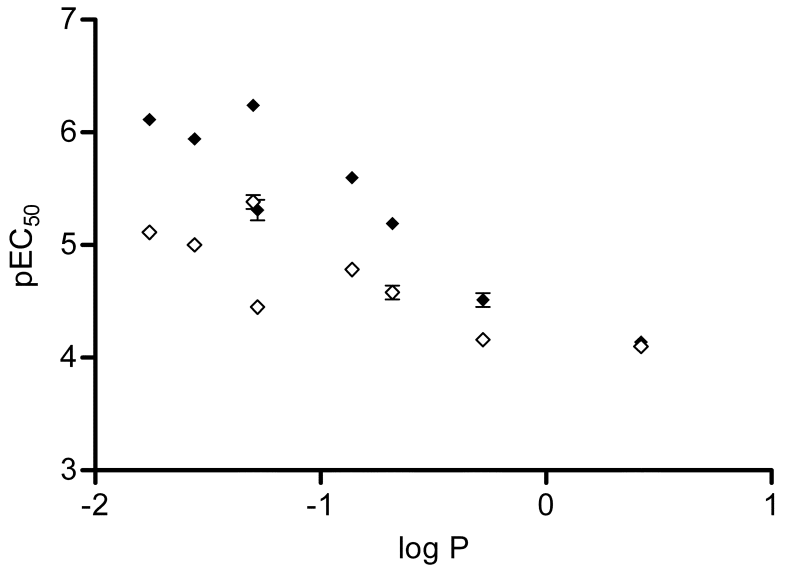


*Fig. 4-22 Relationship between lipophilicity (log P values) and cytotoxicity (pEC_{50} values, n = 3 - 11, mean ± SE) of oxaliplatin and **4** - **10** in HCT-8 (♦) and HCT-8ox (◊) cells.*

The log P values of the oxaliplatin analogues inversely correlated with resistance factors (r = -0.929, p = 0.001) (Fig. 4-23), resulting in a loss of resistance against platinum compounds with positive log P values. Additionally, resistance of HCT-8ox cells against platinum complexes with low reactivity was reduced. Resistance against complexes with high reactivity is at least comparable to resistance against oxaliplatin or even lower (Fig. 4-24).

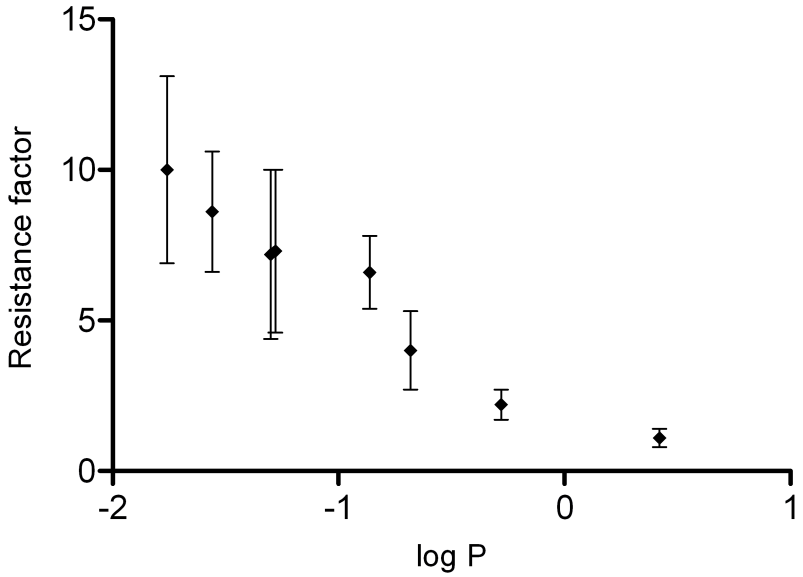


*Fig. 4-23 Relationship between lipophilicity (log P values) and resistance factors (n = 3 - 11, mean ± SD) of oxaliplatin and **4** - **10.***

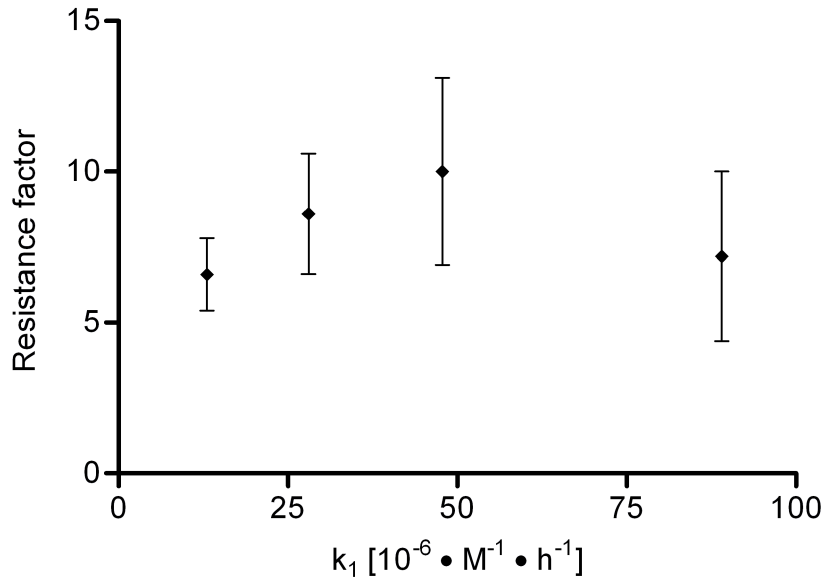


*Fig. 4-24 Relationship between reactivity (k_1 values) and resistance factors (n = 3 - 11, mean ± SD) of oxaliplatin and **8** - **10.***

4.3 Role of transport proteins

4.3.1 Platinum accumulation

To estimate the contribution of passive diffusion to the influx of oxaliplatin, intracellular platinum concentration was determined as a function of the oxaliplatin concentration administered to the cell culture medium and as a function of time (Fig. 4-25 and Fig. 4-26).

Fig. 4-25 shows that the intracellular platinum concentration increased linearly with the concentration of oxaliplatin in the medium (linear regression analysis: HCT-8: r = 0.9996, HCT-8ox: r = 0.9994).

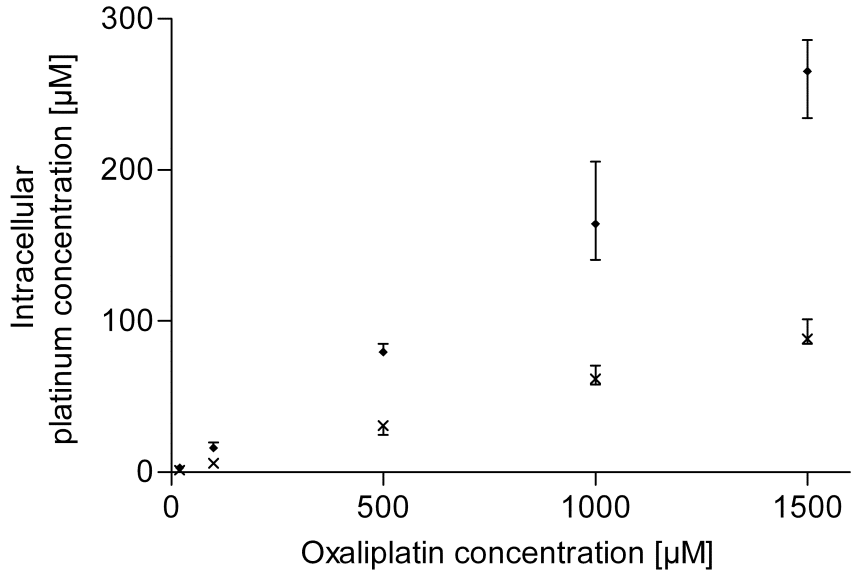


Fig. 4-25 Platinum accumulation after incubation of HCT-8 (♦) and HCT-8ox (x) cells with various concentrations of oxaliplatin for 2 h (n = 6, median ± IQR; some error bars are smaller than data points).

The intracellular platinum concentration in HCT-8ox cells was reduced to 35 % (at 1.5 mM extracellular oxaliplatin) compared to HCT-8 cells.

As shown in Fig. 4-26, intracellular platinum concentration also increased linearly with time (linear regression analysis: HCT-8: r = 0.9996, HCT-8ox: r = 0.9961).

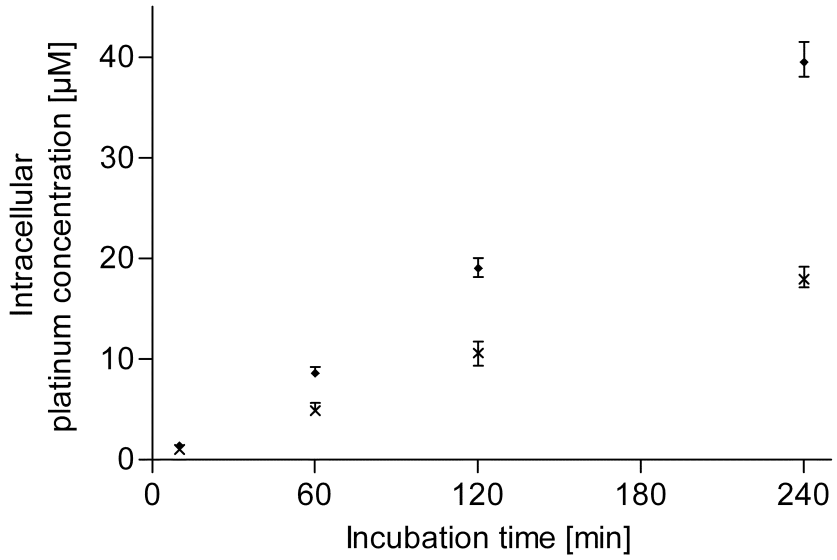


Fig. 4-26 Platinum accumulation after incubation of HCT-8 (♦) and HCT-8ox (x) cells with 100 µM oxaliplatin up to 4 h (n = 6, median ± IQR; some error bars are smaller than data points).

A reduction of the intracellular platinum concentration in HCT-8ox cells to 46 % (at 2 h of incubation) compared to HCT-8 cells was also found.

To investigate the influence of hCTR1 and hOCT1-3, various inhibitors of the transporters or their substrates were used (Tab. 4-7). As hCTR1 is the main copper importer, competitive inhibition of the influx of oxaliplatin by copper sulfate could be expected, if oxaliplatin is a substrate of hCTR1 as well. During coincubation with 100 µM copper sulfate a reduction of the platinum accumulation was detected in both cell lines. Co-exposure to 50 µM copper sulfate did not influence the platinum accumulation. Coincubation with TEA as a substrate of hOCT1-3 caused an impaired accumulation of platinum in both cell lines at the highest TEA concentration (10 mM) and at 100 µM TEA in HCT-8 cells. To further investigate which organic cation transporters might be responsible for this effect, various inhibitors were used. The hOCT1 inhibitor atropine reduced the platinum accumulation only in HCT-8 cells during coincubation and even after preincubation. Cimetidine, known to mainly inhibit hOCT2, led to a decreased platinum accumulation in both cell lines during coincubation as well as after preincubation. The hOCT3 inhibitor decynium-22 did not influence the accumulation of platinum, neither in HCT-8 nor in HCT-8ox cells.

Tab. 4-7 Platinum accumulation in HCT-8 and HCT-8ox cells, respectively, after pre- or coincubation of different substrates and inhibitors of hCTR1 and hOCT1-3 and oxaliplatin (2 h of incubation) as a percentage of the platinum accumulation after incubation with oxaliplatin alone (n = 6 - 12, median (IQR); Mann Whitney U test).

	HCT-8		HCT-8ox	
	Platinum accumulation [%]	**p value**	**Platinum accumulation [%]**	**p value**
Oxaliplatin, 100 µM, 2 h	100	n.a.	100	n.a.
+ Copper sulfate				
coincubation, 100 µM	78.7 (74.3 – 86.2)	0.0020	71.3 (64.8 – 75.3)	0.0028
coincubation, 50 µM	103.5 (96.4 – 110.2)	n.s.	98.9 (93.9 – 105.3)	n.s.
+ TEA				
coincubation, 10 mM	65.9 (57.3 – 66.8)	0.0022	77.6 (73.5 – 86.9)	0.0260
coincubation, 100 µM	69.7 (63.2 – 76.4)	0.0152	84.9 (76.4 – 95.3)	n.s.
coincubation, 1 µM	78.9 (68.2 – 89.9)	n.s.	85.4 (74.6 – 100.7)	n.s.
+ Atropine				
coincubation, 1 mM	72.1 (64.2 – 83.9)	0.0120	99.9 (91.1 – 108.9)	n.s.
preincubation, 1 mM, 1 h	81.4 (68.5 – 92.5)	0.0106	85.2 (79.9 – 93.3)	n.s.
+ Cimetidine				
coincubation, 1.5 mM	73.8 (70.5 – 82.7)	0.0002	74.1 (71.1 – 95.2)	0.0360
preincubation, 1.5 mM, 1 h	78.4 (74.1 – 83.8)	0.0016	78.4 (74.1 – 83.8)	0.0017
+ Decynium-22				
coincubation, 1 µM	94.1 (91.5 – 102.8)	n.s.	93.9 (86.4 – 104.4)	n.s.
coincubation, 0.1 µM	96.8 (91.8 – 111.5)	n.s.	113.2 (83.3 – 121.6)	n.s.

4.3.2 Cytotoxicity

Following influx experiments, the influence of the various inhibitors (or transporter substrates) on cytotoxicity of oxaliplatin was examined. Representative sigmoidal dose-response curves are shown in Fig. 4-27, Fig. 4-28, Fig. 4-29, Fig. 4-30 and Fig. 4-31.

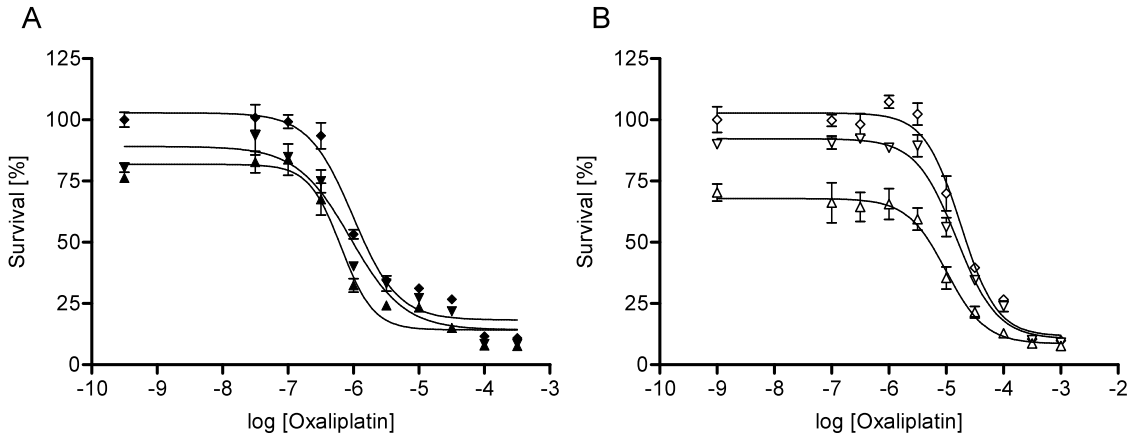


*Fig. 4-27 Representative sigmoidal dose-response curves of oxaliplatin in **A** HCT-8 and **B** HCT-8ox: oxaliplatin alone (♦, ◊), coincubation with 100 µM copper sulfate (▲, △) and coincubation with 50 µM copper sulfate (▼, ▽). Survival is expressed in terms of % of survival of untreated cells.*

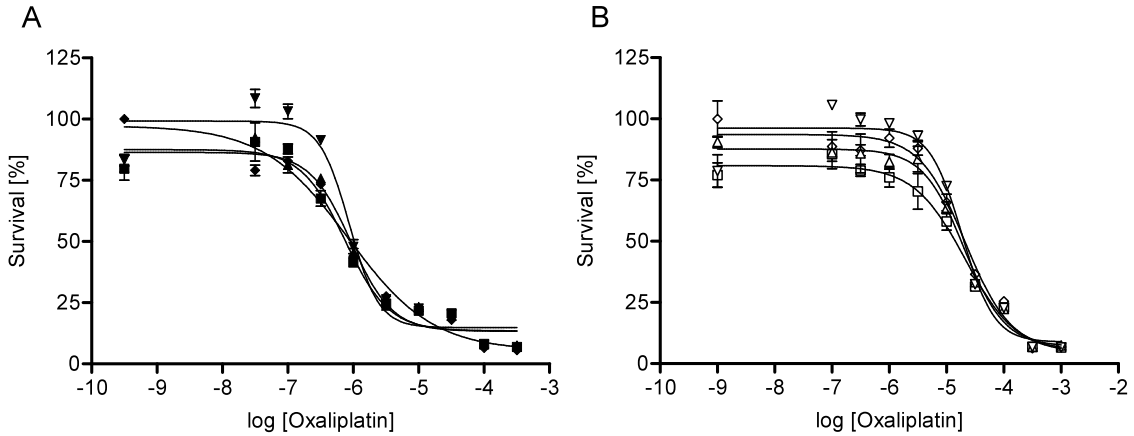


*Fig. 4-28 Representative sigmoidal dose-response curves of oxaliplatin in **A** HCT-8 and **B** HCT-8ox: oxaliplatin alone (♦, ◊), coincubation with 10 mM TEA (■, □), with 1 mM TEA (▲, △) and with 10 µM TEA (▼, ▽). Survival is expressed in terms of % of survival of untreated cells.*

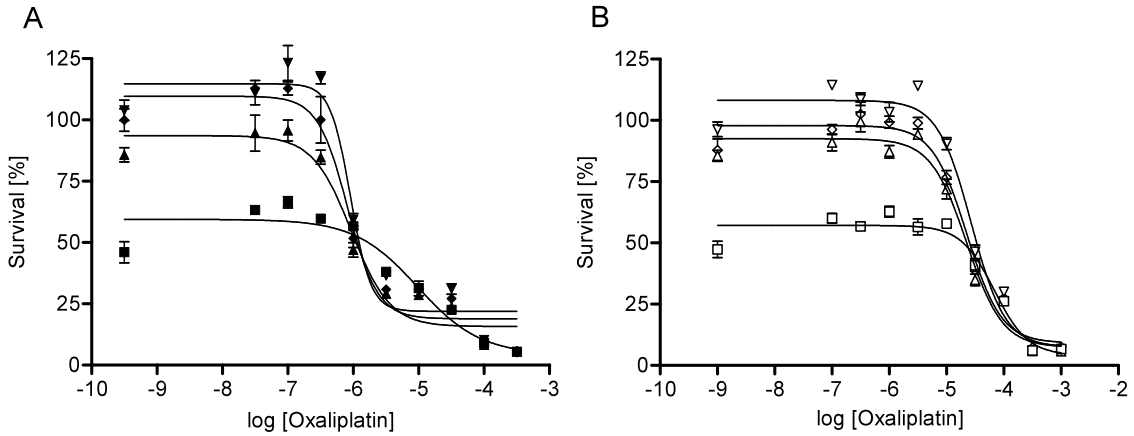


*Fig. 4-29 Representative sigmoidal dose-response curves of oxaliplatin in **A** HCT-8 and **B** HCT-8ox: oxaliplatin alone (♦, ◊), preincubation with 1 mM atropine for 1 h and subsequent incubation with oxaliplatin alone (▲, △), coincubation with 1 mM atropine (■, □) and coincubation with 0.1 mM atropine (▼, ▽). Survival is expressed in terms of % of survival of untreated cells.*

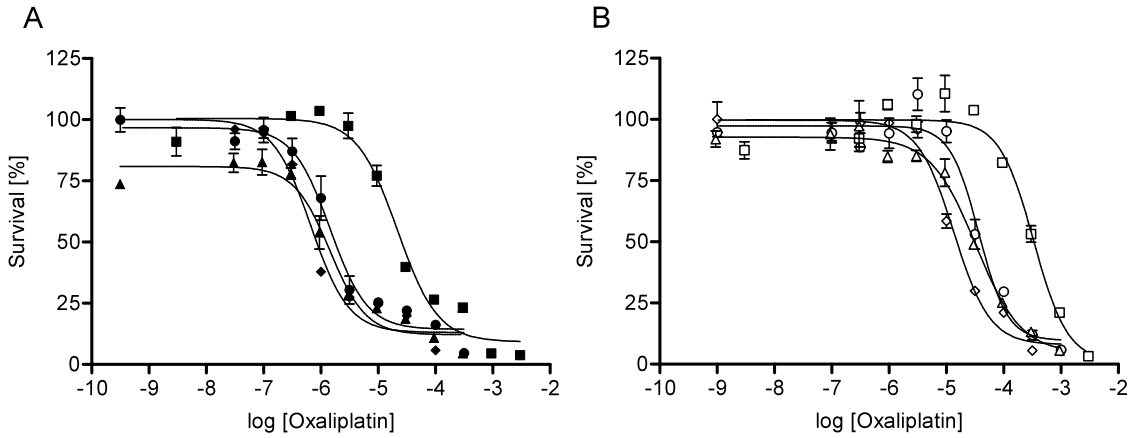


*Fig. 4-30 Representative sigmoidal dose-response curves of oxaliplatin in **A** HCT-8 and **B** HCT-8ox: oxaliplatin alone (♦, ◊), preincubation with 1.5 mM cimetidine for 6 h and subsequent incubation with oxaliplatin alone (●, ○), coincubation with 1.5 mM cimetidine (■, □) and coincubation with 0.15 mM cimetidine (▲, △). Survival is expressed in terms of % of survival of untreated cells.*

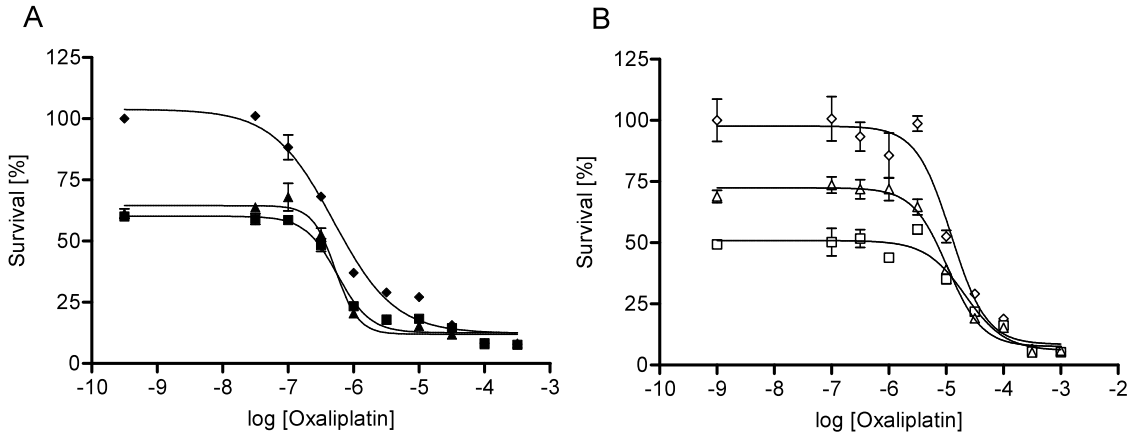


*Fig. 4-31 Representative sigmoidal dose-response curves of oxaliplatin in **A** HCT-8 and **B** HCT-8ox: oxaliplatin alone (♦, ◊), coincubation with 1 µM decynium-22 (■, □) and coincubation with 0.1 µM decynium-22 (▲, △). Survival is expressed in terms of % of survival of untreated cells.*

Based on the sigmoidal dose-response curves pEC_{50} values were calculated. The pEC_{50} values of oxaliplatin and the influence of substrates or inhibitors of hCTR1 and hOCT1-3 on sensitivity of HCT-8 and HCT-8ox cells to oxaliplatin are presented in Tab. 4-8. HCT-8ox cells were found highly resistant to oxaliplatin with a resistance factor of 20.

Copper sulfate, TEA and decynium-22 did not affect cytotoxicity of oxaliplatin. However, coincubation with 1 mM atropine and 1.5 mM cimetidine significantly reduced the cytotoxicity of oxaliplatin.

Tab. 4-8 pEC_{50} values of oxaliplatin alone in comparison with pEC_{50} values of oxaliplatin after pre- or coincubation with different substrates or inhibitors of hCTR1 and hOCT1-3 (copper sulfate, TEA, atropine, cimetidine, decynium-22) in HCT-8 and HCT-8ox (n = 3, mean ± SE; unpaired Student's t-test).

	HCT-8		HCT-8ox	
	pEC_{50} (EC_{50})	**p value**	**pEC_{50}** (EC_{50})	**p value**
Oxaliplatin	6.08 ± 0.03 (0.8 µM)	n.a.	4.77 ± 0.03 (16.8 µM)	n.a.
+ Copper sulfate				
coincubation, 100 µM	6.14 ± 0.06 (0.7 µM)	n.s.	4.87 ± 0.07 (13.6 µM)	n.s.
coincubation, 50 µM	6.15 ± 0.10 (0.7 µM)	n.s.	4.83 ± 0.02 (14.6 µM)	n.s.
+ TEA				
coincubation 10 mM	6.06 ± 0.04 (0.9 µM)	n.s.	4.63 ± 0.06 (23.4 µM)	n.s.
coincubation, 1 mM	6.07 ± 0.02 (0.9 µM)	n.s.	4.76 ± 0.03 (17.3 µM)	n.s.
coincubation, 10 µM	6.12 ± 0.01 (0.8 µM)	n.s.	4.76 ± 0.04 (17.4 µM)	n.s.
+ Atropine				
coincubation, 1 mM	5.06 ± 0.18 (8.7 µM)	0.0045	4.26 ± 0.05 (54.9 µM)	0.0040
coincubation, 0.1 mM	6.08 ± 0.04 (0.8 µM)	n.s.	4.72 ± 0.08 (19.1 µM)	n.s.
preincubation, 1 mM, 1 h	6.04 ± 0.04 (0.9 µM)	n.s.	4.79 ± 0.11 (16.1 µM)	n.s.
+ Cimetidine				
coincubation, 1.5 mM	4.66 ± 0.02 (21.9 µM)	0.0002	3.57 ± 0.04 (269.2 µM)	0.0020
coincubation, 0.15 mM	5.80 ± 0.84 (1.6 µM)	n.s.	4.41 ± 0.07 (39.0 µM)	n.s.
preincubation, 1.5 mM, 6 h	5.90 ± 0.06 (1.3 µM)	n.s.	4.56 ± 0.05 (27.4 µM)	n.s.
+ Decynium-22				
coincubation, 1 µM	6.18 ± 0.03 (0.7 µM)	n.s.	4.72 ± 0.07 (19.1 µM)	n.s.
coincubation, 0.1 µM	6.27 ± 0.05 (0.5 µM)	n.s.	4.87 ± 0.06 (13.5 µM)	n.s.

4.3.3 Gene expression profile

The expression levels of hCTR1 and hOCT1-3 were examined in untreated HCT-8 and HCT-8ox cells as well as in both cell lines after treatment with oxaliplatin. Among a set of 9 possible housekeeping genes (Tab. 3-3), the two most stably expressed genes were identified by GENORM[145,146]: β-Actine and GUS. Expression of the transporters was normalized to the expression levels of the respective housekeeping genes, and is presented in a rescaled way so that untreated sensitive cells obtained a normalized expression value of 1 (Fig. 4-32, Fig. 4-33, Fig. 4-34 and Fig. 4-35).

Oxaliplatin concentrations of > 20 µM were avoided in the expression experiments to prevent a possible interference of oxaliplatin with RNA and subsequent reverse transcription and PCR. Furthermore, the concentration used (20 µM) is within the range of those attainable in plasma of patients receiving oxaliplatin therapy (7.8 – 24.7 µM)[44].

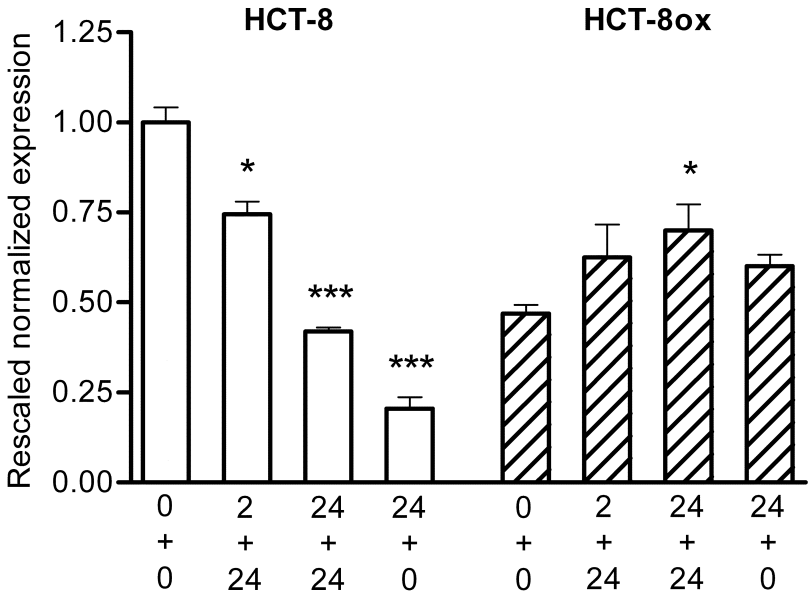


Fig. 4-32 Rescaled normalized expression of hCTR1 in HCT-8 and HCT-8ox cells, not treated with oxaliplatin (0 + 0) and after oxaliplatin treatment (labeling of x axis: first row = duration of treatment in h, second row = duration of subsequent wash-out phase in h). The significance of difference between untreated and treated cells within a single cell line was analyzed (n = 2 - 4, mean ± SE; Tukey´s test, p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)).

The level of hCTR1 was lower in resistant cells than in sensitive cells (Fig. 4-32, $p < 0.001$). Treatment of HCT-8 cells with oxaliplatin resulted in downregulation of hCTR1, already detectable after 2 h of incubation ($p < 0.05$), most lucid after 24 h of incubation without a subsequent wash-out phase ($p < 0.001$). Interestingly, treatment of HCT-8ox cells with oxaliplatin caused a slight upregulation of hCTR1 ($p < 0.05$).

The expression of hOCT1 in untreated HCT-8 and HCT-8ox cells was similar. A significant increase was observed in HCT-8 cells after 24 h of oxaliplatin treatment without a wash-out phase compared to untreated HCT-8 cells (Fig. 4-33). However, no significant differences in hOCT1 expression in HCT-8 and HCT-8ox cells under other treatment conditions compared to the respective untreated cells were observed.

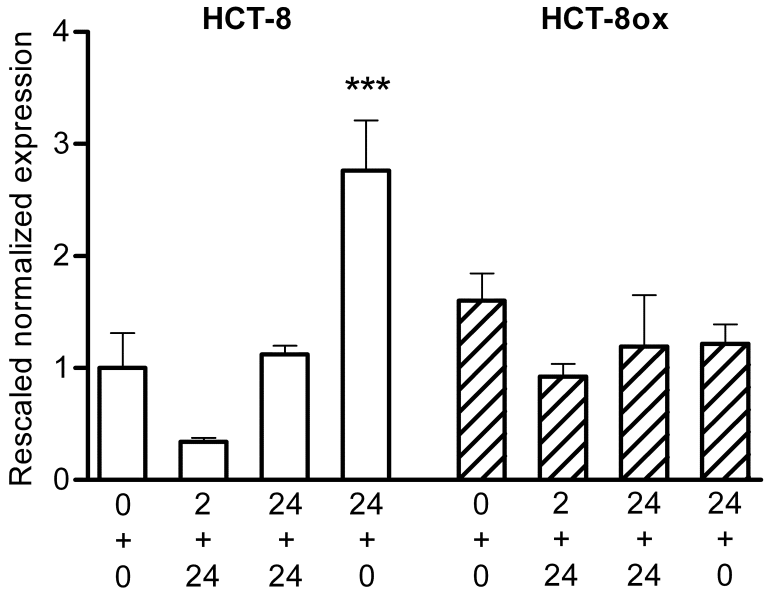


Fig. 4-33 Rescaled normalized expression of hOCT1 in HCT-8 and HCT-8ox cells, not treated with oxaliplatin (0 + 0) and after oxaliplatin treatment (labeling of x axis: first row = duration of treatment in h, second row = duration of subsequent wash-out phase in h). The significance of difference between untreated and treated cells within a single cell line was analyzed (n = 2 - 4, mean ± SE; Tukey´s test, p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)).

The hOCT2 expression in sensitive cells was comparable to that in resistant cells (Fig. 4-34).

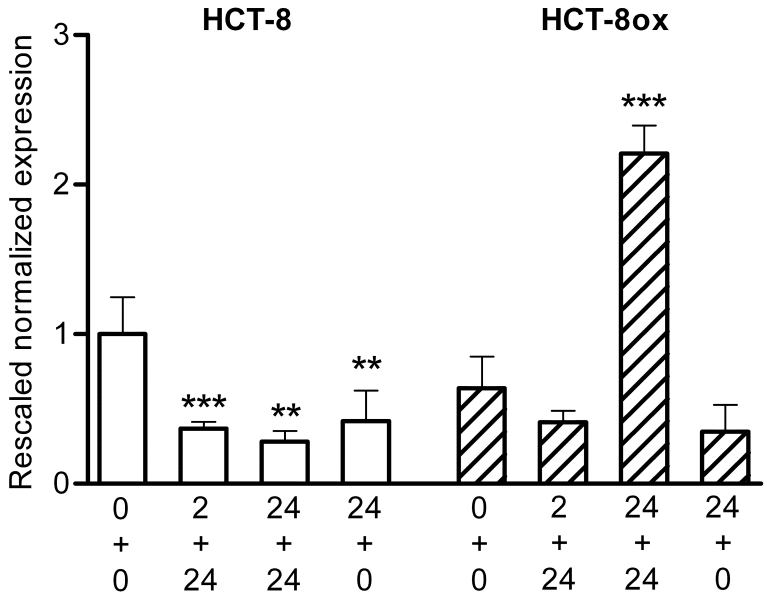


Fig. 4-34 Rescaled normalized expression of hOCT2 in HCT-8 and HCT-8ox cells, not treated with oxaliplatin (0 + 0) and after oxaliplatin treatment (labeling of x axis: first row = duration of treatment in h, second row = duration of subsequent wash-out phase in h). The significance of difference between untreated and treated cells within a single cell line was analyzed (n = 2 - 4, mean ± SE; Tukey´s test, p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)).

Treatment of HCT-8 cells with oxaliplatin resulted in a reduced expression of hOCT2. The contrary was observed in HCT-8ox cells, in which incubation with oxaliplatin for 24 h and a subsequent wash-out phase led to an increased hOCT2 expression. However, no significant changes in the expression level were recognized after 24 h of treatment with oxaliplatin without a wash-out phase.

In HCT-8ox cells the expression of hOCT3 was significantly reduced compared to HCT-8 cells (Fig. 4-35, $p < 0.01$).

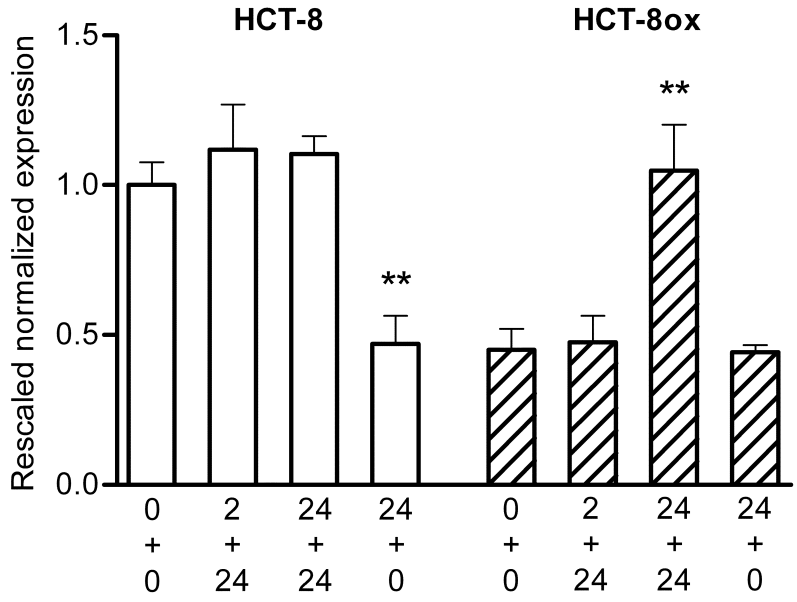


Fig. 4-35 Rescaled normalized expression of hOCT3 in HCT-8 and HCT-8ox cells, not treated with oxaliplatin (0 + 0) and after oxaliplatin treatment (labeling of x axis: first row = duration of treatment in h, second row = duration of subsequent wash-out phase in h). The significance of difference between untreated and treated cells within a single cell line was analyzed (n = 2 - 4, mean ± SE; Tukey´s test, p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)).

Furthermore, the expression of hOCT3 in HCT-8 cells was reduced after treatment with oxaliplatin over 24 h. However, no changes were observed after 2 and 24 h of incubation and a subsequent wash-out phase. During the wash-out phase of 24 h the effect of oxaliplatin on the gene expression seemed to be reversed. In HCT-8ox cells the expression of hOCT3 was increased after 24 h of oxaliplatin treatment and a subsequent wash-out phase. Surprisingly, no changes were determined after 24 h of incubation without a wash-out phase.

In summary, the expression of hCTR1 and hOCT3 was significantly lower in untreated resistant cells than in sensitive cells. Treatment with oxaliplatin influenced the expression of all the transporters studied, but in a different way.

4.3.4 Subcellular localization of transporters

To further investigate the role of hCTR1 and hOCT1-3 in the influx of oxaliplatin and their contribution to resistance, the subcellular localization of the transporters in untreated and oxaliplatin-treated HCT-8 and HCT-8ox cells was analyzed using confocal laser-scanning microscopy after immunohistochemical staining. The concentration of oxaliplatin used in these experiments was within the range of those attainable in plasma of patients receiving oxaliplatin therapy (7.8 – 24.7 µM)[44]. Representative images are shown in Fig. 4-36, Fig. 4-38, Fig. 4-40 and Fig. 4-42. The results are summarized in Tab. 4-9. To support the interpretation of the fluorescence images brightfield images are shown (Fig. 4-37, Fig. 4-39, Fig. 4-41, Fig. 4-43).

In HCT-8 cells hCTR1 was located in both plasma membrane and cytoplasm (Fig. 4-36, Fig. 4-37). Treatment with oxaliplatin did not influence the localization of the transporter. In untreated HCT-8ox cells hCTR1 was also detectable in the plasma membrane and in the cytoplasm. However, after treatment with oxaliplatin the protein moved close to the nucleus and a subsequent wash-out phase caused a redistribution in the direction of the plasma membrane.

In HCT-8 cells the human organic cation transporter hOCT1 was located in the cytoplasm (Fig. 4-38, Fig. 4-39). Oxaliplatin did not change the distribution of the hOCT1 protein. In HCT-8ox cells hOCT1 was partially found in plasma membrane and also cytoplasm. After oxaliplatin treatment the protein was no longer detectable in the plasma membrane. A subsequent wash-out phase provoked a redistribution to the plasma membrane.

Subcellular localization of human organic cation transporter hOCT2 was comparable in sensitive and resistant cells (Fig. 4-40, Fig. 4-41). In both untreated cell lines the protein was dispersed throughout the cytoplasm. After treatment with oxaliplatin hOCT2 was found close to the nucleus. After a subsequent wash-out phase the protein was again present throughout the cytoplasm.

hOCT3 was localized throughout the cytoplasm and in the plasma membrane in both sensitive and resistant cells (Fig. 4-42, Fig. 4-43). Treatment with oxaliplatin did not influence the localization of this protein.

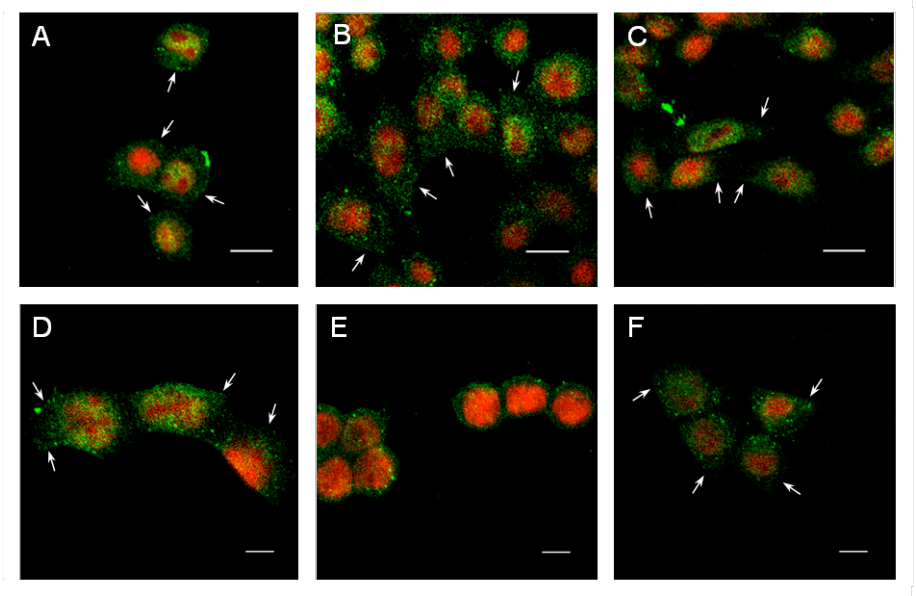


*Fig. 4-36 Immunofluorescence localization of hCTR1 (green) in HCT-8 (**A**, **B**, **C**) and HCT-8ox (**D**, **E**, **F**) without oxaliplatin treatment (**A**, **D**), after 2 h of incubation with oxaliplatin (**B**, **E**), after a subsequent wash-out phase of 2 h (**C**, **F**). Arrows indicate hCTR1 located in the plasma membrane. Nuclei were stained with propidium iodide (red). Scale bars represent 10 µm.*

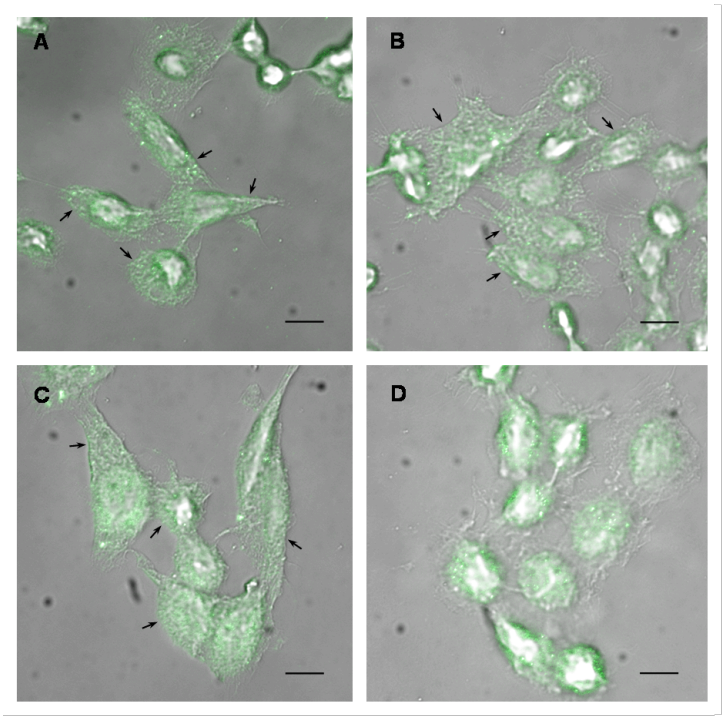


*Fig. 4-37 Immunofluorescence localization of hCTR1 (green) in HCT-8 (**A**, **B**) and HCT-8ox (**C, D**) cells without oxaliplatin treatment (**A**, **C**), after 2 h of incubation with oxaliplatin (**B**, **D**). Arrows indicate hCTR1 located in the plasma membrane. Scale bars represent 10 µm.*

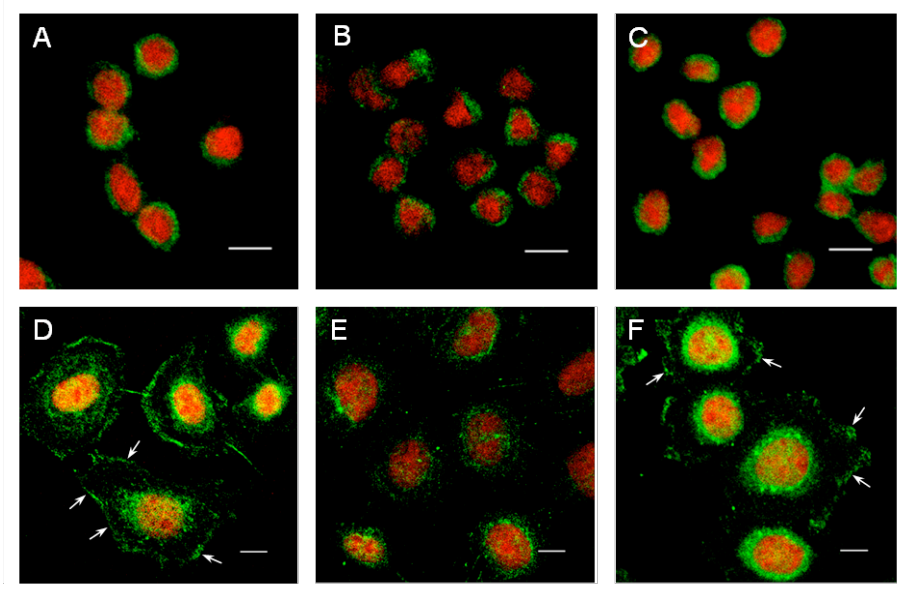


*Fig. 4-38 Immunofluorescence localization of hOCT1 (green) in HCT-8 (**A**, **B**, **C**) and HCT-8ox (**D**, **E**, **F**) without oxaliplatin treatment (**A**, **D**), after 2 h of incubation with oxaliplatin (**B**, **E**), after a subsequent wash-out phase of 2 h (**C**, **F**). Arrows indicate hOCT1 located in the plasma membrane. Nuclei were stained with propidium iodide (red). Scale bars represent 10 µm.*

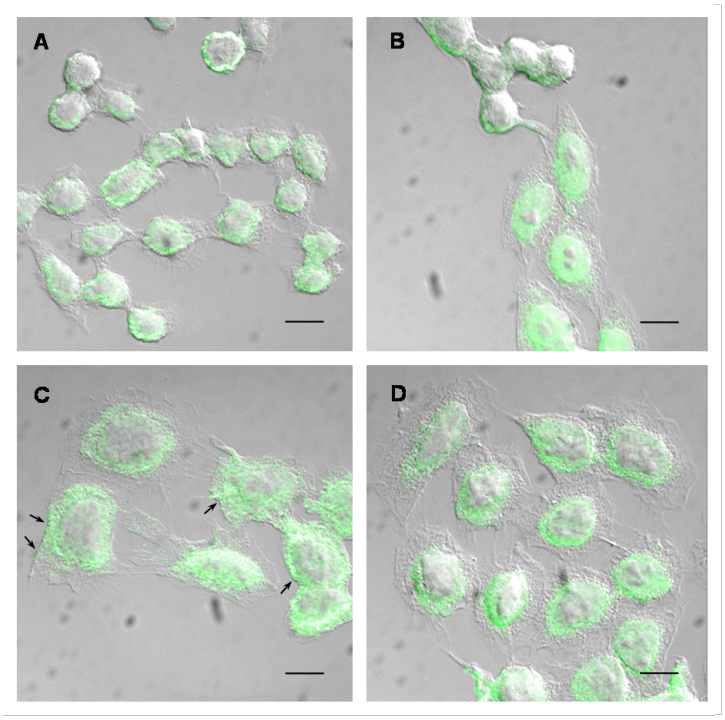


*Fig. 4-39 Immunofluorescence localization of hOCT1 (green) in HCT-8 (**A**, **B**) and HCT-8ox (**C, D**) cells without oxaliplatin treatment (**A**, **C**), after 2 h of incubation with oxaliplatin (**B**, **D**). Arrows indicate hOCT1 located in the plasma membrane. Scale bars represent 10 µm.*

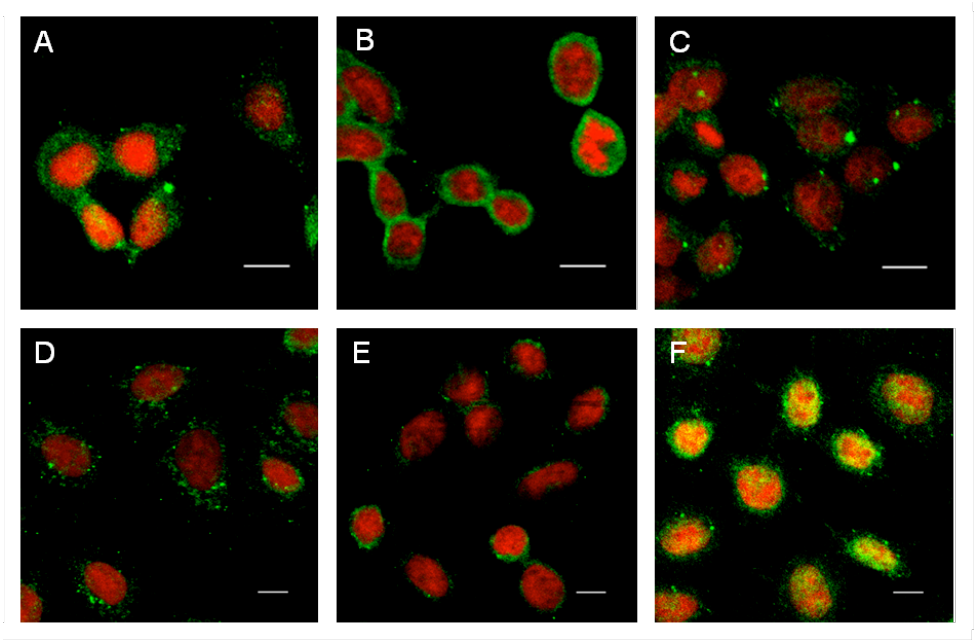


*Fig. 4-40 Immunofluorescence localization of hOCT2 (green) in HCT-8 (**A**, **B**, **C**) and HCT-8ox (**D**, **E**, **F**) without oxaliplatin treatment (**A**, **D**), after 2 h of incubation with oxaliplatin (**B**, **E**), after a subsequent wash-out phase of 2 h (**C**, **F**). Nuclei were stained with propidium iodide (red). Scale bars represent 10 µm.*

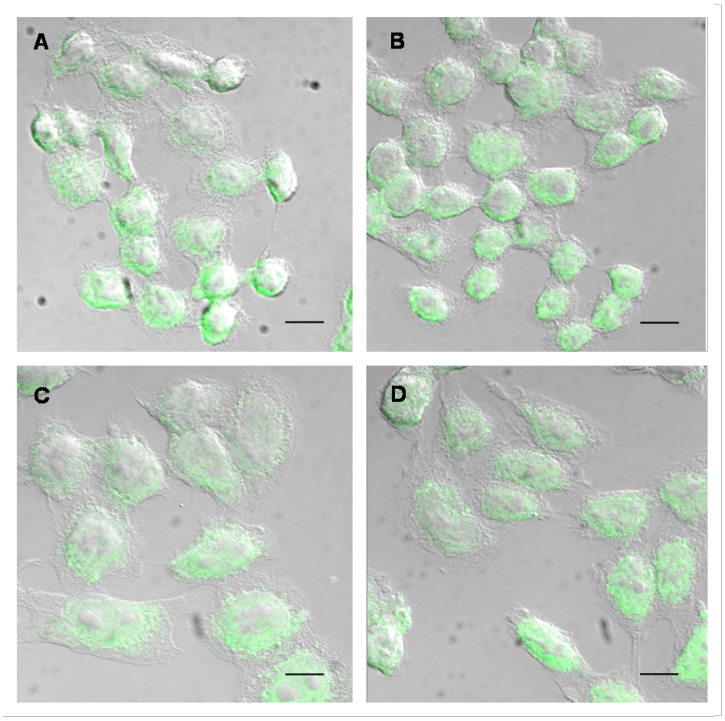


*Fig. 4-41 Immunofluorescence localization of hOCT2 (green) in HCT-8 (**A**, **B**) and HCT-8ox (**C, D**) cells without oxaliplatin treatment (**A**, **C**), after 2 h of incubation with oxaliplatin (**B**, **D**). Scale bars represent 10 µm.*

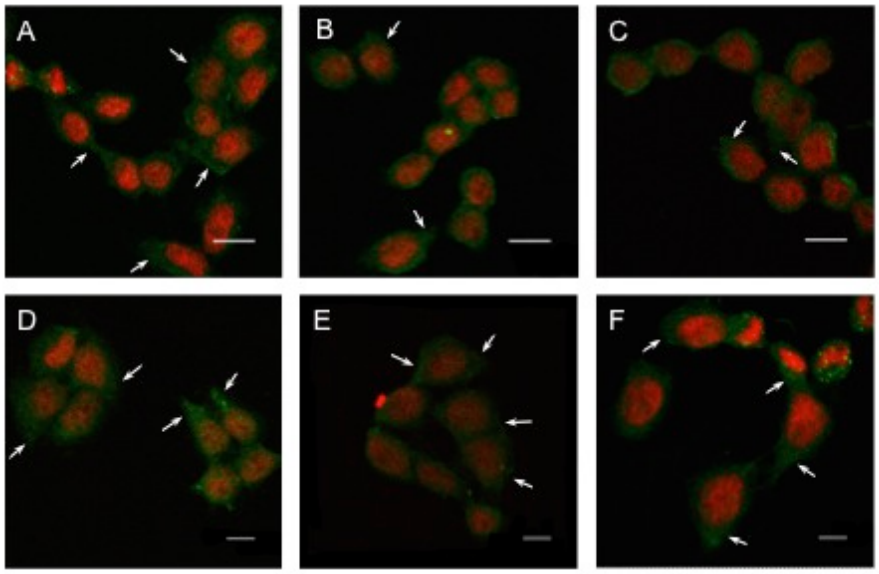


*Fig. 4-42 Immunofluorescence localization of hOCT3 (green) in HCT-8 (**A**, **B**, **C**) and HCT-8ox (**D**, **E**, **F**) without oxaliplatin treatment (**A**, **D**), after 2 h of incubation with oxaliplatin (**B**, **E**), after a subsequent wash-out phase of 2 h (**C**, **F**). Arrows indicate hOCT3 located in the plasma membrane. Nuclei were stained with propidium iodide (red). Scale bars represent 10 µm.*

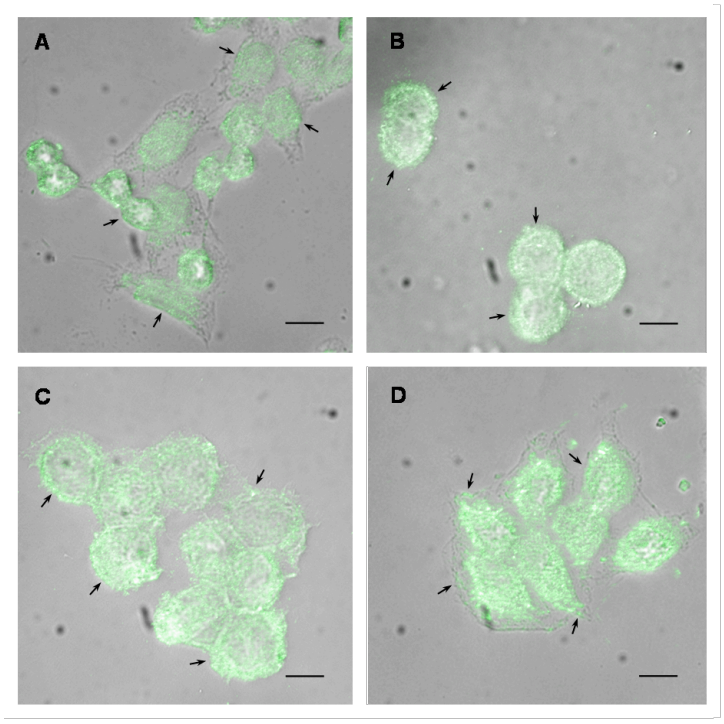


*Fig. 4-43 Immunofluorescence localization of hOCT3 (green) in HCT-8 (**A**, **B**) and HCT-8ox (**C, D**) cells without oxaliplatin treatment (**A**, **C**), after 2 h of incubation with oxaliplatin (**B**, **D**). Arrows indicate hOCT3 located in the plasma membrane. Scale bars represent 10 µm.*

A summary of the subcellular localization of the investigated proteins is given in Tab. 4-9.

Tab. 4-9 Overview of the subcellular localization of hCTR1 and hOCT1-3 in HCT-8 and HCT-8ox cells without treatment of oxaliplatin (- oxaliplatin), after incubation with oxaliplatin (+ oxaliplatin) and after incubation with oxaliplatin followed by a wash-out phase (+ wash-out).

Protein	Treatment	HCT-8	HCT-8ox
hCTR1	- oxaliplatin	plasma membrane, cytoplasm	plasma membrane, cytoplasm
	+ oxaliplatin		close to the nucleus
	+ wash-out		plasma membrane, cytoplasm
hOCT1	- oxaliplatin	cytoplasm	plasma membrane, cytoplasm
	+ oxaliplatin		cytoplasm
	+ wash-out		plasma membrane, cytoplasm
hOCT2	- oxaliplatin	cytoplasm, dispersed	cytoplasm, dispersed
	+ oxaliplatin	close to the nucleus	close to the nucleus
	+ wash-out	cytoplasm, dispersed	cytoplasm, dispersed
hOCT3	- oxaliplatin	plasma membrane, cytoplasm	plasma membrane, cytoplasm
	+ oxaliplatin		
	+ wash-out		

4.4 Interaction between oxaliplatin and cimetidine

4.4.1 Cytotoxicity

Firstly, the effect of cimetidine on cytotoxicity of oxaliplatin was studied (Tab. 4-10).

Tab. 4-10 pEC_{50} values of oxaliplatin alone in comparison with pEC_{50} values of oxaliplatin after pre- or coincubation with cimetidine in HCT-8 and HCT-8ox cells (n = 3, mean ± SE; unpaired Student´s t-test).

	HCT-8		HCT-8ox	
	pEC_{50} (EC_{50})	**p value**	**pEC_{50}** (EC_{50})	**p value**
Oxaliplatin	5.99 ± 0.05 (1.0 µM)	n.a.	4.69 ± 0.06 (20.4 µM)	n.a.
Preincubation, 6 h	5.90 ± 0.06 (1.3 µM)	n.s.	4.56 ± 0.05 (27.5 µM)	n.s.
Preincubation, 12 h	5.91 ± 0.07 (1.2 µM)	n.s.	4.59 ± 0.05 (25.7 µM)	n.s.
Coincubation	4.66 ± 0.02 (21.9 µM)	0.0002	3.57 ± 0.04 (269.2 µM)	0.0020

Pretreatment with cimetidine for 6 and 12 h did not affect the cytotoxic potency of oxaliplatin. However, coincubation significantly reduced cytotoxicity of oxaliplatin in both HCT-8 and HCT-8ox cells.

4.4.2 Long-term influx experiments

4.4.2.1 Protein determination

In the long-term influx experiments intracellular platinum concentration should be expressed in ng platinum/mg protein, because a calculation based on a fixed number of cells was not appropriate due to the growing of the cells during the experiment. It is commonly known that a linear relationship between protein and cell concentration exists. However, it had to be assured that the conditions of the preparation of the samples were chosen in a way, which guaranteed a complete lysis of the cells even at high cell concentrations.

Addition of 1 mM sodium hydroxide and subsequent sonication of the samples for 30 min turned out to lyse the cells completely (Fig. 4-44).

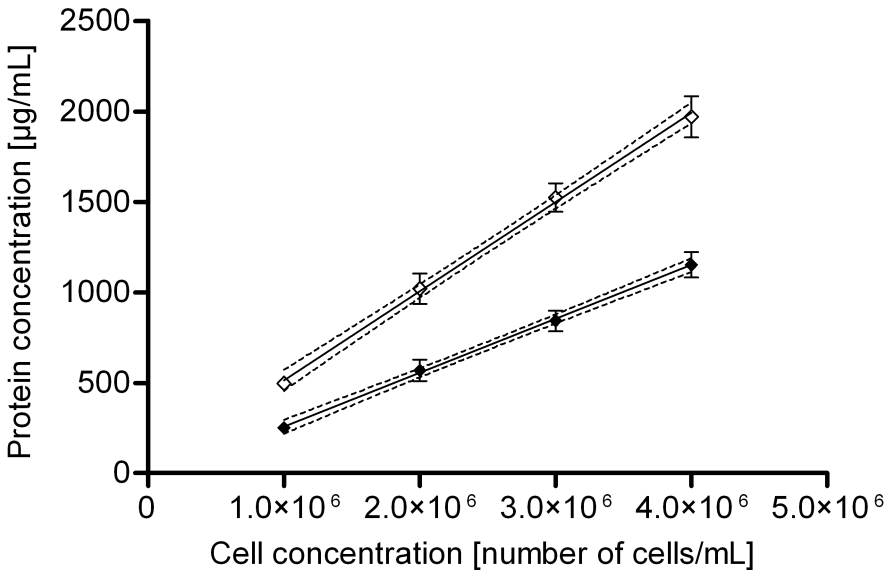


Fig. 4-44 Relationship between cell and protein concentration in HCT-8 (♦) and HCT-8ox (◊) cells (calibration curve: continuous line; 95 % confidence interval: broken line; n = 6, mean ± SD).

A linear relationship between protein and cell concentration was observed. The mean protein content of a sensitive cell was 276 ± 29 pg and of a resistant cell 502 ± 31 pg. Within-day precision ranged between 3.4 and 14.3 % and between-day precision between 5.2 and 13.6 % (see Appendix D2).

4.4.2.2 Influx experiments

In accordance with the results of the determination of cytotoxicity, a reduction of the intracellular platinum accumulation during coincubation, but not during preincubation with cimetidine was observed (Fig. 4-45).

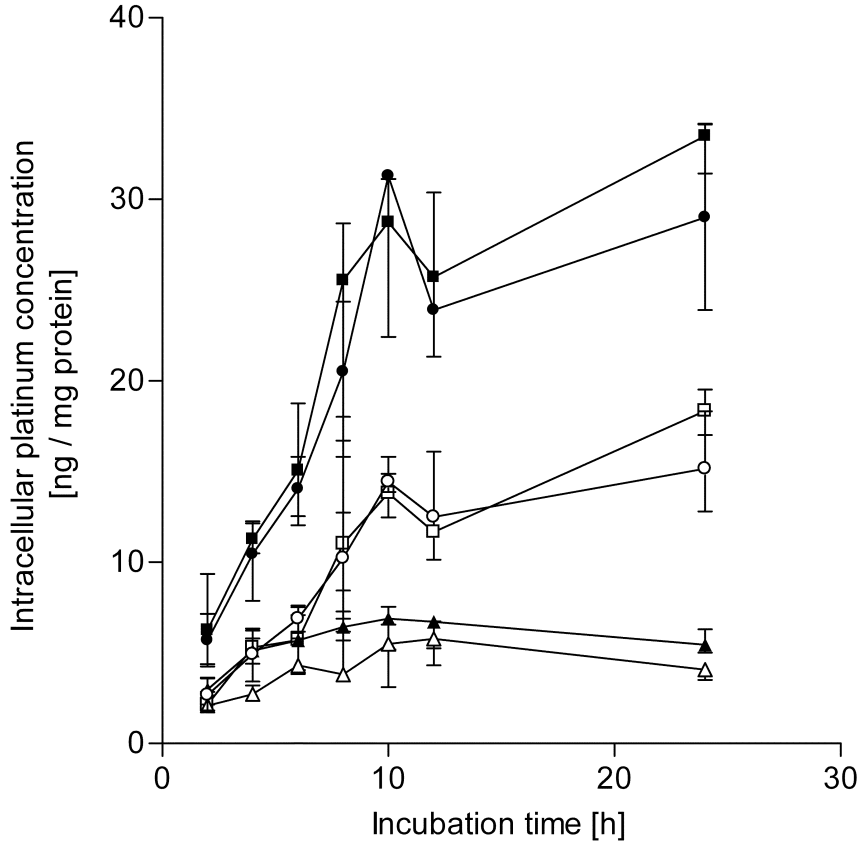


Fig. 4-45 Intracellular platinum concentration after incubation with 20 µM oxaliplatin up to 24 h (■, HCT-8, □, HCT-8ox), after coincubation with 20 µM oxaliplatin and 1.5 mM cimetidine (▲, HCT-8, △, HCT-8ox) and after preincubation with 1.5 mM cimetidine for 12 h and subsequent incubation with 20 µM oxaliplatin alone (●, HCT-8, ○, HCT-8ox), (n = 3-9, median ± IQR; some error bars are smaller than data points).

Since preincubation with cimetidine had no effect and coincubation significantly affected intracellular platinum concentrations and cytotoxicity, it was presupposed that oxaliplatin could react with cimetidine in culture medium, thereby resulting in the reduced cellular accumulation and hence in reduced cytotoxicity.

4.4.3 Chemical interaction between oxaliplatin and cimetidine

Monitoring the reaction between oxaliplatin and cimetidine showed that interaction between the compounds was fast, as no peak of oxaliplatin at δ = – 1970 ppm could be detected already after 1 h. Instead, a peak at δ = – 3245 ppm corresponding to the product of the reaction was found (Fig. 4-46).

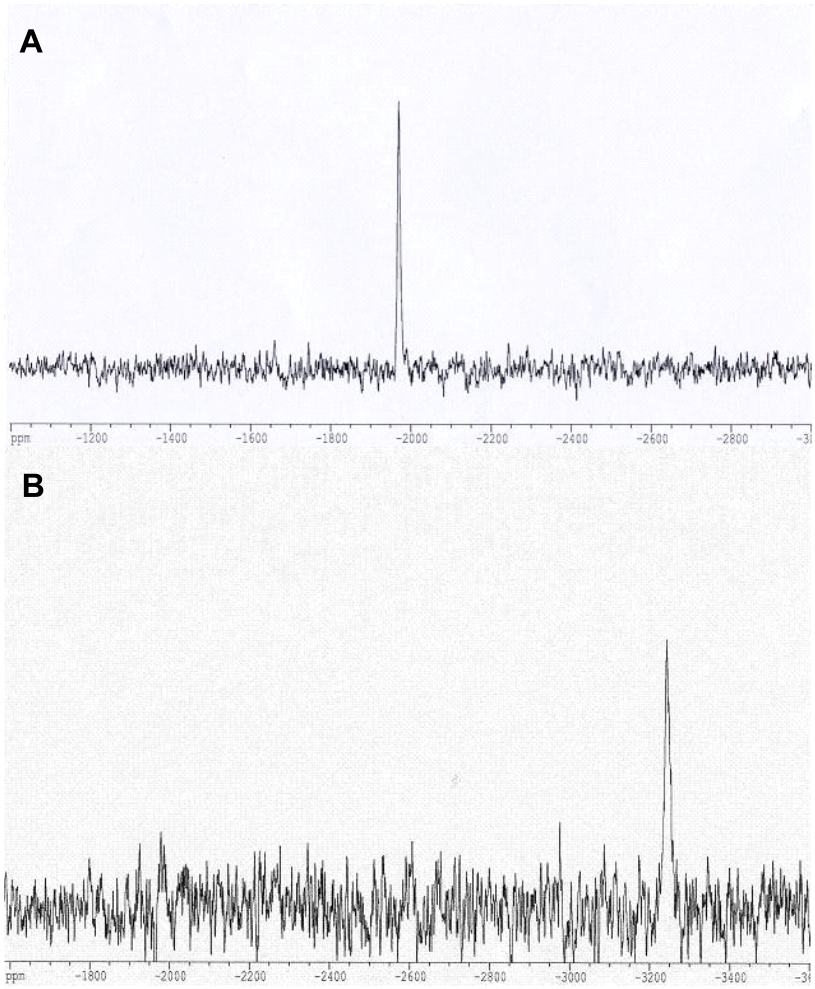


*Fig. 4-46 ^{195}Pt NMR spectrum of 10 mM oxaliplatin in PBS/DMF (1:1) at 37 °C (**A**) and ^{195}Pt NMR spectrum of a mixture of 10 mM oxaliplatin and 150 mM cimetidine in PBS/DMF at 37 °C after coincubation for 1 h (**B**).*

5 Discussion

In chapter 5.1 and 5.2 the results of the systematic investigation on the relationships between lipophilicity, reactivity, influx and cytotoxicity of oxaliplatin and its analogues in human carcinoma cells are discussed. In chapter 5.3 the role of the transporters hCTR1 and hOCT1-3 in influx and cellular processing of oxaliplatin and in oxaliplatin resistance is elucidated.

5.1 Oxaliplatin analogues with different amine ligands

5.1.1 Influx

The first objective of this investigation was to reveal whether it is possible to overcome one of the main oxaliplatin resistance mechanisms – a reduced influx – by enhancing the lipophilicity of the complex. For this purpose, the influence of lipophilicity on the influx kinetics of oxaliplatin analogues was assessed in different tumor cell lines. Reactivity towards nucleophiles is also supposed to play a major role in the influx, especially in the influx mediated by CTR1[108,110]. Despite possessing the same leaving group, differences in reactivity of the investigated platinum complexes were possible due to altered steric hindrance. However, all investigated oxaliplatin analogues showed a comparable reactivity. Therefore, it had to be assumed that differences in cellular accumulation are mainly related to different lipophilicity of platinum complexes. Nonetheless, the influence of other so far unknown properties of platinum complexes with relevance for influx cannot be excluded.

Within the first minutes of incubation a correlation between log P values and influx rates was observed, suggesting a lipophilicity-dependent early influx phase. Most likely passive diffusion of the complexes in or across the plasma membrane dominates in the early influx phase.

Passive diffusion should still proceed in the late influx phase, however with a reduced rate because of the reduced concentration gradient. In fact, the late influx rate was lower than the early influx rate, but no longer determined by lipophilicity. It was therefore supposed that reactivity mainly influences the influx rate. This may

correspond to a reactivity-dependent binding of the platinum complexes to transport proteins, e.g. CTR1 or OCT1-3[3,54,147]. In the resistant cell line a reduction of the influx rate, even in the early influx phase, was found. As previously described for the ovarian carcinoma cell line (A2780/A2780cis) this may be associated with the reduced expression of CTR1[148].

In contrast, the late influx rate of the most lipophilic platinum compound **7** was increased in the resistant cell line compared to the sensitive counterpart while efflux of **7** was not altered. Therefore, the higher accumulation may be a result of an increased influx. If lipophilicity of this complex is the only reason for increased late influx rate, the influx rate in the sensitive cell line would also be increased, which was not the case. One may speculate that the biophysical properties of the plasma membrane of resistant cells are altered leading to a favored transport of lipophilic complexes in or across the membrane. Changes in the membrane composition of the resistant cell lines have been associated with reduced influx[3,54]. In a cisplatin-resistant cell line lower membrane fluidity due to a tighter packing of phospholipid molecules, an altered dynamic character of lipid molecules and a lower index of unsaturated fatty acids were observed[149]. On the contrary, more “fluid” plasma membranes were found in another cisplatin-resistant cell line[150]. The changes appear to be cell line specific and their contribution to resistance has been discussed controversially.

5.1.2 Cytotoxicity

The increased accumulation of the more lipophilic complexes found in each cell line leads to the question whether it is possible to increase the cytotoxic potency of a platinum complex in a given cell line by increasing lipophilicity.

Given the observed differences in the accumulation of the platinum complexes in the early influx phase, an increase in cytotoxicity with increasing lipophilicity could be expected. This has already been described for other platinum complexes[116,117,151]. Surprisingly, an inverse correlation between lipophilicity and cytotoxicity was found in both cell lines. A lower cytotoxic potency of the complexes with bulky substituents at position 4 of the cyclohexane ring was previously reported in different human cancer cell lines[124,125]. In order to understand the reduced cytotoxic potency of the more

lipophilic complexes the intracellular fate of oxaliplatin and **7** as the least and the most lipophilic complexes was investigated in more detail.

The reduced cytotoxicity of **7** was accompanied by a reduced DNA platination compared with oxaliplatin although the cellular accumulation of this complex was higher. On the one hand, changes in reactivity of the platinum complexes with bulky substituents once they have entered the cell cannot be completely excluded. The reduced DNA platination might be a result of a reduced reactivity of **7** towards DNA compared to the reactivity towards single nucleotides. On the other hand, this observation might suggest that **7** is trapped in the cytoplasm, either by binding to nucleophiles and therefore inactivation or by sequestration in subcellular compartments[152-155]. There is already evidence for sequestration of lipophilic platinum complexes. A platinum complex with a lipophilic anthraquinone carrier ligand was found to accumulate in lysosomes. It has been discussed that amphiphilicity of this platinum complex leads to partitioning in the plasma membrane without diffusion through the membrane followed by internalization by endosomes and accumulation in lysosomes[152]. Mitochondria may represent another cellular organelle capable of sequestration of platinum complexes. The increased mitochondrial membrane potential in tumor cells compared to normal epithelial cells could result in an increased accumulation of lipophilic cations[156]. According to a predictive model, strong bases and permanent cations with intermediate lipophilicity (log P between -2 and 2 with an optimum at 0) tend to accumulate in mitochondria of tumor cells[157]. Although there is some evidence of an involvement of lysosomes and mitochondria in sequestration of platinum complexes, the exact subcellular localization of **7** needs further clarification.

To summarize, increasing lipophilicity of oxaliplatin by introducing substituents to the DACH carrier ligand failed to enhance cytotoxicity in the investigated cell lines.

5.1.3 Resistance

In a previous study a correlation between the expression of copper transporter 1, intracellular platinum concentration, DNA platination and cell death in the ovarian carcinoma cell line pair (A2780/A2780cis) was observed[148]. Resistance of A2780cis

cells to cisplatin was mainly based on a reduced intracellular platinum concentration due to a reduced influx, which was followed by reduced DNA platination[148].

Consequently, a circumvention of the reduced influx should result in a diminution of resistance[114,115]. In the ileocecal colorectal adenocarcinoma cell line pair **7** was able to bypass the reduced influx in the resistant cell line. To estimate the impact of the increased influx of **7** on DNA platination and resistance, these processes were quantified and compared with those after oxaliplatin treatment (Tab. 5-1).

Tab. 5-1 Contribution of cellular accumulation and DNA platination to resistance.

	HCT-8ox vs. HCT-8	
	Oxaliplatin	**7**
Fold resistance	10.0	1.1
Fold decrease in accumulation after 2 h	1.8	0.6
Fold decrease in DNA platination	2.4	1.6

In the ileocecal colorectal adenocarcinoma cell line pair the expected correlation between intracellular platinum concentration, DNA platination and resistance was observed. In HCT-8ox cells a decreased platinum-DNA adduct formation was observed upon a decreased accumulation after treatment with oxaliplatin. However, other processes might be involved, as the resistance factor was rather high. No significant repair of platinum-DNA adducts in both ileocecal colorectal adenocarcinoma cell lines was found. Thus it is entirely conceivable that enhanced adduct tolerance represents a second resistance mechanism in this cell line, which has often been detected in cells with acquired resistance to oxaliplatin[57]. A closer look to the relationship between influx rate of **4**, **5** and **6** and the resistance factor showed that despite their lower accumulation in HCT-8ox cells compared to their sensitive counterpart, the resistance factor diminished with increasing lipophilicity. These results suggest that reduction of resistance was either due to decreased inactivation of oxaliplatin analogues or mediated by mechanisms occurring after DNA binding. For instance, cellular processing of the platinum-DNA adducts generated by more lipophilic as well as bulkier carrier ligands might be altered in this cell line pair. Differences in the processing of cisplatin- and oxaliplatin-DNA adducts are well

known, e.g. the failure of recognition of the more lipophilic and bulkier oxaliplatin-DNA adducts by proteins of the MMR system and other damage recognition proteins like HMG domain proteins[4,7,158]. Thus, an influence of the substituents of the DACH carrier ligand on cellular processing is conceivable.

5.2 Oxaliplatin analogues with different leaving groups

5.2.1 Reactivity

Until now a broad spectrum of nucleophiles has been used to quantify the reactivity of platinum complexes, e.g. G-actin[110], L-methionine[159,160], glutathione[96,96,159,161], calf thymus DNA[104,162] and guanosine-5'-monophosphate[159,160,163]. The choice of the model compound depends on the underlying question. For the investigation of the relationship between reactivity and cytotoxicity, the determination of reactivity towards nucleophiles seems to be appropriate. However, it is more difficult to identify an appropriate model compound for investigating the influence of reactivity on cellular accumulation, because of a lack of knowledge about the involved transport mechanism(s). Some evidence exists that the copper transporter hCTR1 contributes to the influx of platinum complexes, especially to the influx of cisplatin[3]. However, a contribution of hCTR1 to the influx of oxaliplatin at concentrations higher than 2 µM is unlikely[67]. Therefore, the assessment of reactivity was restricted to the reaction between the platinum compound and nucleotides as targets inside the cell. In general, one should keep in mind that the reactivity depends on the nucleophile as well as on the conditions under which the reaction takes place[160].

The reduced reactivity of all platinum complexes towards dAMP compared to dGMP is well documented[164]. The degree of reactivity of the complexes is determined by the structure of the leaving group and steric aspects. **8** might be less reactive than cisplatin because of the steric hindrance of the amine ligand. The formation of a chelate complex increases the stability, which might explain the reduced reactivity of oxaliplatin, **9** and **10** compared to **8**[165]. The higher the electron density of the leaving group, the less reactive the complexes are: oxalate > malonato > cyclobutane-1,1-dicarboxylato. The high stability and, therefore, low reactivity of the complexes with a cyclobutane-1,1-dicarboxylato leaving group is well known[109,163]. The reactivity of platinum(IV) compounds depends on their reduction potential. The binding is accelerated by an increase in the reduction potential and the ease of reduction depends on the axial ligands with X = Cl > OAc > OH[166-168]. Therefore, the inertness of **11** and **12** might be a consequence of their low reduction potential[168].

5.2.2 Influx

According to the previous results, more lipophilic complexes should enter the cells more rapidly in the early influx phase, in which lipophilicity is the rate-determining influx mechanism. Furthermore, the hypothesis that more reactive compounds should enter the cells faster than inert compounds by triggering protein-mediated transport was generated.

By increasing lipophilicity the early influx rate increased, further strengthening the above drawn conclusion (see 5.1). Most likely, passive diffusion dominates in the early influx phase. The results of the correlation analysis also point to a contribution of passive diffusion in the late influx phase in HCT-8ox cells. Passive diffusion is a continuously ongoing process only slowed down by a decreasing concentration gradient. From this point of view it is obvious to detect an influence of lipophilicity also on the late influx rate. However, influence of lipophilicity was negligible in the sensitive cell line indicating the existence of another influx mechanism.

Reactivity is supposed to be an important factor in the influx via a lipophilicity-independent mechanism. In fact, beside lipophilicity, reactivity of the complexes influenced both early influx rate and late influx rate. Increasing reactivity led to a higher influx rate: **10** < oxaliplatin < **8** (the influx of **9** will be discussed below). A comparable relationship was also determined during incubation with oxaliplatin, $Pt(DACH)Cl_2$ and $Pt(DACH)(H_2O)_2$[92]. However, the exact role of the exchange of the leaving ligand in the influx of platinum complexes still remains unanswered, because it is unknown which platinum species are taken up by the cell. Either the ligand exchange already occurs within the cell culture medium and reactive chloro or aqua complexes are formed, which are transported into the cell, or the exchange occurs by direct interaction with proteins in the plasma membrane responsible for the influx of platinum complexes [108,109,160]. The up to 50 % reduced influx of most of the compounds in the resistant cell line might be a result of both a downregulation of transport proteins and changes in the membrane lipid composition [3].

The most lipophilic oxaliplatin analogue **9** deserves closer examination. Despite the low reactivity, cellular accumulation of **9** was relatively high and even higher in the resistant than in the sensitive cell line. To evaluate the influence of the structure of

the amine ligand and leaving group, the cellular accumulation of **9**, the least reactive oxaliplatin analogue, and carboplatin as well as **8**, the most reactive oxaliplatin analogue, and cisplatin was compared. Interestingly, the concentration-time profiles of cisplatin and **8** were similar, suggesting a minor influence of the amine ligand. Reactivity seems to be crucial for the influx. On the contrary, the concentration-time profiles of carboplatin and **9** were completely different, in spite of bearing the same leaving group. In this case lipophilicity has an exceptionally high impact on the influx of **9** compared to reactivity. This property apparently allows the complex to bypass the resistance mechanism, which leads to a reduced influx of the other investigated complexes. In the first part of this study of oxaliplatin analogues with different amine ligands a similar behavior of the most lipophilic compound **7** was observed. A change in the biophysical properties of the plasma membrane of the resistant cells leading to a favored transport of lipophilic complexes in or across the membrane was suggested. This might not only explain the influence of lipophilicity on the late influx phase in the resistant cells but also the high accumulation of **9**. The comparison of the cellular accumulation of these complexes demonstrates the relative importance of lipophilicity versus reactivity.

Unlike **9**, the cellular accumulation of the platinum(IV) complexes (**11** and **12**) was very low. A low cellular accumulation of platinum(IV) compounds with axial hydroxo ligands, especially of **11**, was already described[167,169]. Either the hydroxo ligand itself affects the influx or a low reduction potential is responsible for the reduced influx[169]. A high reduction potential accelerates the influx, however, a preceding reduction in the extracellular medium does not seem to be essential for the influx[154,167,170]. Therefore, a small amount of **11** still enters the cell. Regarding the influx of **12**, a deprotonation of the axial ligand at physiological pH was presumed to be responsible for the low cellular accumulation of this complex[119]. These negatively charged species are possibly not able to permeate the plasma membrane.

5.2.3 Cytotoxicity and resistance

Cytotoxic potency was influenced by lipophilicity and reactivity. High lipophilicity led to a reduced cytotoxicity, despite an increased influx of more lipophilic complexes in the early influx phase. The reason might be an impaired formation of platinum-DNA

adducts by lipophilic platinum complexes, which could already be demonstrated for oxaliplatin and **7**. In this second part of this study it was demonstrated that an increase in reactivity leads to a higher cytotoxicity, maybe due to a higher influx. In summary, if lipophilicity of the investigated oxaliplatin analogues is moderate, reactivity seems to be the most decisive property. Thus, based on these results, lipophilicity of the compound should be as low as possible and reactivity as high as possible for a high cytotoxic potential.

Nevertheless, the more lipophilic oxaliplatin analogues had the potential of overcoming resistance. Regarding the platinum complexes with different amine ligands (oxaliplatin and **4** – **7**) a different recognition and processing of the platinum-DNA adducts due to their different structure was proposed. The platinum(II) compounds oxaliplatin and **8** – **10** possess the same amine ligand. Consequently, the structure of the platinum-DNA adducts will probably be the same. Therefore, reactions occurring before, and not after DNA binding might be more relevant.

Which processes taking place before binding to the DNA might be altered in the resistant cells? First of all, a reduction of the influx might contribute to resistance. However, no correlation between the fold decrease in accumulation after 2 h and resistance factors was observed. Therefore, changes in the influx might only contribute to a minor extent to resistance. Processes and reactions occurring in the cytoplasm might also participate in the emergence of resistance. As already discussed, increased sequestration of lipophilic platinum complexes might be responsible for the reduced cytotoxicity (see 5.1). Hence, diminished sequestration of the lipophilic complexes in the resistant cells might increase the amount of platinum compound reaching the DNA and therefore reduce the resistance factor. Changes in cellular organelles in resistant tumor cells such as reduction of the lysosomal compartment to 40 % and an altered cellular distribution and function of mitochondria have been described and may contribute to the lower degree of resistance against lipophilic compounds[171,172].

Beside lipophilicity, reactivity also represents an important property of platinum complexes regarding the reactions occurring in the cytoplasm. An increased inactivation by sulfur-containing molecules, like glutathione, metallothionein, but also sulfur-containing high molecular mass molecules, is commonly assumed to represent

one of the major resistance mechanisms of tumor cells[5]. Accordingly, less reactive platinum complexes are expected to be less affected and platinum complexes of higher reactivity are expected to be more affected by metabolic reactions. Consequently, resistance of the investigated cell line pair against less reactive platinum complexes was lower than resistance against more reactive compounds. Therefore, in order to overcome resistance lipophilicity of the platinum compounds should be high, presumably a log P value of approximately 0, and reactivity should be low. The slightly reduced resistance of cells against **8** also points at the ability of highly reactive complexes to overcome resistance. However, further investigations are needed to confirm this hypothesis.

Now the question arises, which properties should a platinum compound possess to exhibit a well-balanced relationship between cytotoxic potency and toxicity and to overcome the resistance mechanisms in cancer cells at the same time. According to the results of the experiments with the oxaliplatin analogues the log P value should be approximately 0 and reactivity should be low. Though compounds of low reactivity are less cytotoxic, they bear the advantage of producing less side effects[103]. If the hypothesis that also highly reactive compounds might be able to overcome platinum resistance turns out to be true a platinum complex with a 4-tert-butyl-cyclohexane-1,2-diamine amine ligand and two chloro leaving groups might be a good candidate for optimization studies.

In the following, the results of the experiments with the platinum(IV) compounds are discussed. Despite the low reactivity and influx, the platinum(IV) complexes developed a certain cytotoxicity, which was even comparable to the cytotoxic potential of carboplatin. That a low cellular accumulation does not necessarily result in a low cytotoxicity, was also demonstrated with platinum(IV) compounds having the general structure DACH-Pt(IV)-X_2Y_2 with X = Cl, OAc or TFA as axial ligands and Y = Cl as equatorial ligand[173]. Although a direct interaction of platinum(IV) compounds with DNA or nucleotides was described, the intracellular reduction seems to be crucial for the cytotoxicity of the complexes[173-175]. Resistance of HCT-8ox cells against **11** and **12** was lower than against oxaliplatin and **8** – **10**, but a direct comparison is not possible because of the structural differences.

5.3 Role of transport proteins

It is reported here on the contribution of passive diffusion as well as protein-mediated transport on the influx of oxaliplatin. The investigations were focused on the copper transporter 1 (hCTR1) and the organic cation transporters 1-3 (hOCT1-3). When indicated, the contribution of these transporters to reduced influx and to resistance was elucidated. An overview of the key points of the discussion is presented in Tab. 5-2.

5.3.1 Passive diffusion

The data show that oxaliplatin enters the cells partly via passive diffusion. The hallmarks of passive diffusion were observed in the cell system: influx was linear, concentration dependent and nonsaturable within a wide concentration range. However, there are some arguments against passive diffusion as the only influx mechanism. Firstly, accumulation of oxaliplatin was reduced in resistant cells. This might be caused by changes in the plasma membrane composition, though it is doubtful that these changes provoke a reduction in the influx to about 50 %[3]. The second argument for the involvement of other transport mechanisms are the results of the investigation of the structure-transport relationship (see 5.1 and 5.2), which point at a protein-mediated transport of oxaliplatin.

5.3.2 Copper transporter hCTR1

The localization of hCTR1 in the plasma membrane of both sensitive and resistant cells possibly points at the contribution of hCTR1 to the influx of oxaliplatin. Furthermore, the reduced platinum accumulation after coincubation of oxaliplatin and copper might be provoked by mutual inhibition of the transport of copper and oxaliplatin or by degradation of hCTR1 following copper exposure. Although treatment with extensive concentrations of copper was followed by a marked reduction of hCTR1 levels in the plasma membrane in transfected cell lines, this observation could not be verified in cell lines endogenously expressing hCTR1[72,108,176-178]. Therefore, a mutual inhibition of the transport of copper and oxaliplatin is likely. Further, due to the reduced cellular accumulation of platinum,

reduced cytotoxicity of oxaliplatin during coincubation with copper sulfate was also expected. However, this was not the case. A possible explanation is that the fraction of oxaliplatin, which has been transported by hCTR1, does not reach its pharmacological target, the DNA[72]. One might even speculate that the binding of oxaliplatin to the Met-rich extracellular domain of hCTR1 is already irreversible[45,163,179]. On the other hand, the effect of coincubation with copper sulfate on the influx of oxaliplatin might have been not sufficient to affect cytotoxicity of oxaliplatin. An indirect evidence of contribution of hCTR1 to influx and/or cellular processing of oxaliplatin has been adduced by investigation of the influence of oxaliplatin on subcellular localization and gene expression of hCTR1. Both alterations in localization of hCTR1 in HCT-8ox cells and in expression in HCT-8 and HCT-8ox cells during incubation with oxaliplatin hint at the involvement of hCTR1 in oxaliplatin transport. Redistribution of hCTR1 in HCT-8ox cells might be partly responsible for reduced influx of the drug in these cells compared to sensitive cells. Additionally, the a priori reduced expression of hCTR1 in resistant cells may contribute to the reduced influx. The increased mRNA expression in resistant cells after oxaliplatin treatment may be caused by the need of the cells to have sufficient amounts of copper. Because hCTR1 disappeared from the plasma membrane in HCT-8ox cells upon oxaliplatin exposure, the copper supply might be hindered. Therefore, HCT-8ox cells might counteract by upregulation of the expression of hCTR1[180,181]. Taken together, the data point out at the relevance of hCTR1 for reduced influx of oxaliplatin in HCT-8ox cells. However, contribution of the transporter to resistance in this cell line needs further clarification.

5.3.3 Organic cation transporters hOCT1-3

In order to evaluate the influence of organic cation transporters on influx of oxaliplatin the model OCT substrate TEA was used[77]. At the highest concentration of TEA applied in this study the maximum transport rate of hOCT1, hOCT2 and even hOCT3, which has the lowest affinity to TEA, can be achieved[77]. The reduction of oxaliplatin influx in both cell lines after coincubation with TEA let one assume that at least one of these transporters participates in the influx of oxaliplatin. However, the reduced influx did not affect cytotoxicity of oxaliplatin, possibly due to the same

reasons as discussed above, the inability to reach the DNA or an insufficient effect of TEA. The contribution of the each particular transporter was investigated using specific inhibitors.

Atropine inhibits hOCT1 with the highest known affinity[77,78]. The influence of atropine on the influx of oxaliplatin in HCT-8 cells may reflect a contribution of hOCT1 to oxaliplatin transport across the plasma membrane. However, the protein appeared to be not located in the plasma membrane. Nevertheless, hOCT1 might steadily cycle through different compartments of the cells and be localized intracellular by a process of continuous retrieval from the cell surface[178]. The evidence of the contribution of this transporter to influx was further strengthened by the reduced cytotoxicity of oxaliplatin during coincubation with atropine. Nonetheless, it should be noted that survival of the atropine-treated cells was in general diminished. Reduced cytotoxicity of oxaliplatin might simply be a result of the retarded cell growth. This might also explain the reduction of cytotoxic activity of oxaliplatin in HCT-8ox cells, despite an unchanged influx in presence of atropine. Interestingly, in HCT-8ox cells hOCT1 was detected in the plasma membrane. Nevertheless, in contrast to the sensitive cells it is suggested that hOCT1 is not involved in the influx of oxaliplatin in resistant cells, because the influx was not influenced by co-/preincubation with atropine. The absence of oxaliplatin influx via hOCT1 in HCT-8ox cells might contribute to the reduced influx in this cell line compared to the sensitive cell line and contribute to resistance of HCT-8ox cells. Furthermore, the protein may in some way be directly or indirectly involved in cellular processing of oxaliplatin, as indicated by the increased gene expression after treatment of sensitive cells with oxaliplatin and the lucid alterations of the cellular localization of the protein in resistant cells after treatment with oxaliplatin.

The influence of organic cation transporter hOCT2 on influx of platinum drugs has often been studied using cimetidine, which acts mainly as an inhibitor of hOCT2, under the assumption that it does not chemically interact with the platinum drugs[87,88]. However, an interaction between platinum drugs and cimetidine cannot be completely ruled out. Therefore, this question was studied in more detail[182]. Reduced cytotoxicity of oxaliplatin and reduced intracellular platinum accumulation during long-term coincubation of oxaliplatin and cimetidine, but not during long-term

preincubation, could be on the one hand a result of an inhibition of hOCT2 by cimetidine. On the other hand this could also be due to an interaction of oxaliplatin and cimetidine already in the cell culture medium, with the consequence that this new platinum complex is not taken up by the cell. The behavior of a mixture of oxaliplatin and cimetidine was investigated using ^{195}Pt NMR. The chemical shift of the peak indicates a Pt ion to be in an [N_3S] coordination surrounding, suggesting that [Pt(DACH)(cimetidine)]$^{2+}$ was formed (Fig. 5-1).

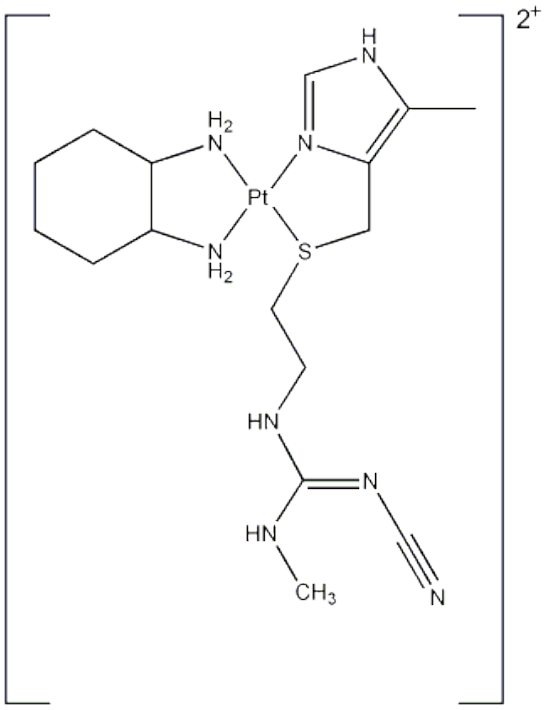


Fig. 5-1 Proposed product of the interaction between oxaliplatin and cimetidine.

Thus, under the conditions of coincubation experiments oxaliplatin is bound to cimetidine. This is the first report about interaction between oxaliplatin and cimetidine under physiological conditions. The data clearly show that the results of the studies, in which a platinum complex is coincubated with a transporter inhibitor, should be treated with caution. For example, a recent publication reported that coincubation with cimetidine significantly reduced oxaliplatin accumulation but did not affect cisplatin and carboplatin accumulation[88]. Given rapid interaction between oxaliplatin and cimetidine, reaction of cisplatin and carboplatin with the inhibitor of hOCT2 is also likely. At first glance, one would expect reduction of cisplatin and carboplatin

accumulation, as well as their cytotoxicity. However, it is possible that the product of reaction of cisplatin and carboplatin with cimetidine $[Pt(NH_3)_2(cimetidine)]^{2+}$ is readily taken up by the cell, whereas $[Pt(DACH)(cimetidine)]^{2+}$ is not. Since organic cation transporters (e.g. OCT1) mostly transport molecules with molecular weight below 500 Da, $[Pt(NH_3)_2(cimetidine)]^{2+}$ (M = 481 Da) might be a better substrate for them than $[Pt(DACH)(cimetidine)]^{2+}$ (M = 561 Da)[77]. However, even if this would be the case, it remains unclear whether $[Pt(NH_3)_2(cimetidine)]^{2+}$ species can platinate genomic DNA and thereby exert cytotoxic action.

Despite the evidence of a direct interaction between oxaliplatin and cimetidine, in short-term experiments not only coincubation but also preincubation leads to diminished influx. Therefore, it is suggested that reduction in the influx of oxaliplatin by cimetidine is a result of direct binding between the substances in the cell culture medium as well as of inhibition of hOCT2-mediated influx. However, the organic cation transporter hOCT2 was not located in the plasma membrane but in an intracellular compartment in both cell lines. For that reason one would exclude initially a contribution of this transporter to the influx of oxaliplatin. It is hypothesized that hOCT2 contributes to the influx of oxaliplatin in both cell lines and that the missing membrane localization might be due to the continuous cycling of the protein through different cellular compartments[178]. Since cytotoxicity of oxaliplatin was only decreased upon coincubation with cimetidine, it was not possible to determine to which extent each effect, binding between the substances in the cell culture medium and inhibition of hOCT2-mediated transport, contributed to the reduced cytotoxicity. Involvement of hOCT2 in the reduced influx in resistant cells seems to be unlikely, because neither the localization nor the gene expression of hOCT2 differed between untreated resistant and sensitive cells. However, there is evidence that hOCT2 may contribute to cellular processing of oxaliplatin, since the localization and the gene expression of the protein changed after treatment with oxaliplatin.

The organic cation transporter hOCT3 was located in the cytoplasm as well as in the plasma membrane in untreated sensitive and resistant cells. However, the hOCT3-inhibitor decynium-22 did neither inhibit the influx of oxaliplatin nor its cytotoxicity, to wit, either (1) the inhibitor was not potent enough or (2) the inhibitor binds hOCT3 in a way that the transport of model cations is inhibited, but the transport of oxaliplatin is

not, or (3) hOCT3 is not involved in the transport of oxaliplatin. The first explanation seems improbable, because decynium-22 is one of the most potent inhibitors of hOCT3[138]. However, due to lack of knowledge regarding mechanisms of influx of platinum complexes via organic cation transporters, the second explanation cannot be ruled out. Therefore, an involvement of hOCT3 should not be precluded until this issue is clarified. The results of the expression analysis imply a contribution of hOCT3 to the transport of oxaliplatin, as the expression of hOCT3 in HCT-8ox cells was reduced compared to HCT-8 cells and the expression levels were influenced by treatment with oxaliplatin.

The key points of the discussion are summarized in Tab. 5-2.

Tab. 5-2 Contribution of hCTR1 and hOCT1-3 to influx, cellular processing and cytotoxicity of oxaliplatin (+, involved; (+), possibly involved; -, not involved).

Protein	Influx		Cellular processing		Resistance
	HCT-8	HCT-8ox	HCT-8	HCT-8ox	
hCTR1	+	+	(+)	(+)	(+)
hOCT1	+	-	(+)	(+)	+
hOCT2	+	+	(+)	(+)	-
hOCT3	(+)	(+)	(+)	(+)	(+)

6 Conclusions and outlook

Within the scope of this study new insights in general influx mechanisms and in transporter-mediated influx of oxaliplatin could be gained. The investigated structure-transport and structure-activity relationships of oxaliplatin analogues serve as a basis for the development of new platinum compounds. However, to manifest the conclusions reached in this study more oxaliplatin analogues should be included in the investigation. Especially compounds of higher reactivity and higher lipophilicity might provide added value.

The examination of the role of transport proteins in influx of oxaliplatin suggest that a number of transporters are probably capable of accommodating oxaliplatin influx. In general, this kind of studies are very dependent on the inhibitors used. Therefore, for future investigations it is desirable to have more specific and potent inhibitors available. Furthermore, investigation of the protein expression and conduction of colocalization studies of fluorescent oxaliplatin analogues and transport proteins might help to clarify the exact role of hCTR1 and hOCT1-3 in influx of oxaliplatin and its contribution to resistance.

To be able to interpret the results of this study in a more sophisticated way, the mechanisms of resistance of the HCT-8/HCT-8ox cell model should be examined in more detail, e.g. investigation of the membrane composition, the degree of sequestration and, more generally, the intracellular inactivation of oxaliplatin analogues and the investigation of the mechanisms occurring after binding to DNA. The transferability of the results to other cell models should be also verified.

In summary, this study makes a valuable contribution to the elucidation of the influx of oxaliplatin and provides ideas for the development of new platinum compounds. Nevertheless, due to the complexity of the reactions taking place within a cell there are still many open questions to be faced.

7 Summary

This thesis describes the first systematic investigation of the relationship between lipophilicity and reactivity on the one hand and cellular accumulation and cytotoxic activity on the other hand of a new class of oxaliplatin derivatives with different substituents at position 4 of the cyclohexane ring and different leaving ligands. Furthermore, the contribution of hCTR1 and hOCT1-3 on the influx of oxaliplatin was investigated. Information regarding the influence of oxaliplatin on the expression of these transporters, as well as on their localization inside cancer cells is still limited. In contrast to the studies conducted in the past, in this study no transfected cell lines were used.

Lipophilicity and reactivity are important determinants of the platinum influx. In contrast to the early influx phase, in which lipophilicity predominantly governs the influx rate, the late influx phase is mainly controlled by the reactivity of a platinum compound. Passive diffusion is suggested as the preponderant influx mechanism in the early influx phase and protein-mediated transport as the major influx mechanism in the late influx phase.

Lipophilicity and reactivity are also determinants of the cytotoxic potency of platinum compounds and of tumor resistance. Although lipophilicity enhances the influx of platinum complexes, especially in the early influx phase, an increased lipophilicity leads to a reduction of cytotoxic potency of the investigated oxaliplatin analogues. This might be due to alterations in the reactivity of platinum complexes with bulky substituents in the intracellular environment or increased intracellular sequestration of lipophilic complexes. On the other hand, an increased lipophilicity is associated with the advantage of overcoming resistance. This does not seem to be related to increased influx. Changes in the reactions occuring before binding to the DNA, as well as different recognition and processing of the formed DNA adducts due to the variation of the carrier ligand might be another explanation for this phenomenon which, however, requires clarification in future investigations. Contrary to lipophilicity, an increase in reactivity enhances the cytotoxic potency of a platinum compound. Furthermore, resistance against platinum complexes of low reactivity appears to be reduced, possibly because these compounds are less affected by cytosolic

inactivation. Therefore, a well-balanced relationship between lipophilicity and reactivity may be a starting point for optimization of the oxaliplatin structure. A log P value of approximately 0 and a low reactivity should support (a) passive diffusion as well as (b) the possibility of overcoming resistance.

In any event, passive diffusion represents an important mechanism of oxaliplatin influx. Furthermore, it is supposed that oxaliplatin is transported by hCTR1 and that this transport is reduced in resistant cells. It is questionable whether the thereby decreased influx contributes to resistance. Influx of oxaliplatin via hOCT1 occurs in sensitive cells, however is unlikely in resistant cells. This fact might partly account for the reduced influx of oxaliplatin in resistant cells and contribute to resistance of HCT-8ox cells. hOCT2 is assumed to be involved in the influx of oxaliplatin in both cell lines to a similar extent suggesting no major relevance of this transporter for resistance. It could be demonstrated that cimetidine, routinely used as inhibitor of hOCT2 in influx studies, interacts with oxaliplatin under physiological conditions. The data strongly suggest that the results of studies, in which a platinum complex is coincubated with a transporter inhibitor, should be reinterpreted. hOCT3 may participate in cellular processing of oxaliplatin. However, its involvement in the influx of the platinum drug remains questionable. The relevance of this transporter for oxaliplatin resistance cannot be ruled out, but requires more detailed investigation.

The results of this thesis contribute to the elucidation of influx of platinum compounds and of resistance provoked by reduced influx. Based on these results platinum complexes with improved activity against oxaliplatin-resistant tumor cells can be developed. Additionally, the results may support the efforts to refine individualized chemotherapy by consideration of the expression profile of the investigated transport proteins.

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Appendix

Appendix A

Results of the investigation on oxaliplatin analogues with different amine ligands

A1 Influx

Intracellular platinum concentration [µM] in HCT-8 and HCT-8ox cells after incubation with 100 µM platinum complex up to 120 min.

	HCT-8			HCT-8ox		
			Oxaliplatin			
Time	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
10	1.23	0.29	12	0.63	0.28	18
60	8.17	0.65	12	3.12	1.68	18
120	14.47	1.23	12	7.86	2.97	18
			Compound 4			
10	1.57	0.17	6	0.70	0.29	6
60	5.99	1.00	6	3.44	1.96	6
120	14.28	5.75	6	5.81	2.60	6
			Compound 5			
10	1.33	0.17	6	1.22	0.13	6
60	5.69	0.97	6	5.58	1.51	6
120	14.28	1.49	6	9.64	0.47	6
			Compound 6			
10	3.95	2.31	6	1.90	0.56	6
60	13.57	5.70	6	7.02	3.55	6
120	17.96	0.04	6	10.13	3.31	6
			Compound 7			
10	6.65	1.10	6	3.30	0.81	6
60	11.82	6.70	6	10.50	5.83	6
120	17.20	5.42	6	27.59	5.37	6

n, number of experiments; IQR, interquartile range

Early and late influx rate in HCT-8 and HCT-8ox cells after incubation with 100 µM platinum complex [µM/min].

	Early influx rate		Late influx rate	
Compound	**HCT-8**	**HCT-8ox**	**HCT-8**	**HCT-8ox**
Oxaliplatin	0.12	0.06	0.12	0.08
4	0.16	0.07	0.12	0.05
5	0.13	0.12	0.12	0.08
6	0.40	0.19	0.14	0.07
7	0.67	0.33	0.10	0.22

A2 Efflux

*Intracellular platinum concentration in HCT-8 and HCT-8ox cells after 120 min incubation with oxaliplatin or **7** and subsequent incubation with drug-free medium up to 120 min (n = 6). The median platinum concentration at the end of the 120 min incubation period was set to 100 %.*

	Oxaliplatin				Compound 7			
	HCT-8		HCT-8ox		HCT-8		HCT-8ox	
Time	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
0	100.0	6.5	100.0	4.8	100.0	5.9	100.0	3.3
2	109.6	26.7	110.9	12.2	76.9	7.4	83.0	15.9
5	86.6	36.8	83.5	4.4	53.2	23.7	76.0	3.3
10	85.2	6.6	80.9	11.4	74.7	6.2	79.0	9.2
60	82.1	14.7	80.7	12.0	75.2	9.0	74.8	10.4
120	53.7	29.4	50.6	45.4	68.0	5.1	74.8	32.3

IQR, interquartile range

A3 Cytotoxicity

Survival of HCT-8 and HCT-8ox cells after incubation with different concentrations of platinum complex. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Oxaliplatin								
Untreated	100.0	2.8	0.891	0.025	100.0	9.4	0.449	0.042
-7.5	106.3	1.9	0.947	0.017	--	--	--	--
-7.0	99.6	7.7	0.887	0.069	128.4	14.3	0.577	0.064
-6.5	90.9	5.9	0.809	0.053	134.3	3.8	0.603	0.017
-6.0	53.5	4.3	0.477	0.038	123.0	1.9	0.552	0.009
-5.7	35.8	3.9	0.319	0.035	99.7	6.8	0.448	0.030
-5.3	33.3	2.3	0.288	0.020	89.0	5.9	0.400	0.027
-5.0	29.9	2.8	0.266	0.025	53.7	1.2	0.241	0.005
-4.5	25.0	0.7	0.223	0.006	36.2	1.0	0.163	0.005
-3.5	4.9	0.2	0.044	0.002	11.00	0.3	0.049	0.001
-3.0	--	--	--	--	10.8	0.3	0.048	0.001
Compound 4								
Untreated	100.0	8.2	0.721	0.059	100.0	3.5	0.634	0.022
-7.5	106.4	2.1	0.651	0.015	92.6	2.8	0.587	0.018
-6.5	94.5	5.7	0.630	0.041	94.4	1.0	0.598	0.006
-5.5	56.1	2.2	0.444	0.016	93.0	7.1	0.589	0.045
-5.2	34.7	3.4	0.298	0.025	87.8	6.7	0.556	0.042
-4.9	30.4	1.8	0.237	0.013	78.0	4.4	0.494	0.028
-4.6	23.5	1.4	0.213	0.010	58.4	3.8	0.370	0.024
-4.3	15.1	1.2	0.179	0.009	28.5	4.4	0.180	0.028
-4.1	6.9	0.9	0.100	0.007	9.5	0.4	0.060	0.003
-4.0	6.1	0.5	0.086	0.004	8.8	1.6	0.056	0.010
-3.7	5.4	0.7	0.074	0.005	8.3	2.0	0.052	0.012
Compound 5								
Untreated	100.0	8.4	0.633	0.053	100.00	3.2	0.665	0.021
-7.5	81.7	3.0	0.517	0.019	101.75	2.6	0.677	0.018
-6.5	79.2	16.8	0.501	0.106	91.93	0.9	0.612	0.006
-5.5	66.9	12.1	0.423	0.077	78.56	3.5	0.523	0.023
-5.2	38.5	3.7	0.243	0.023	82.11	1.6	0.546	0.011
-4.9	28.3	4.5	0.179	0.028	59.87	6.2	0.398	0.041
-4.6	19.7	1.2	0.124	0.008	42.08	4.3	0.280	0.029
-4.3	16.5	2.0	0.104	0.013	31.76	1.0	0.211	0.007
-4.1	7.1	0.2	0.045	0.001	18.64	1.2	0.124	0.008
-4.0	6.7	0.6	0.042	0.004	14.58	0.9	0.097	0.006
-3.7	6.7	0.3	0.042	0.002	12.12	1.1	0.081	0.008

(Cont.) Survival of HCT-8 and HCT-8ox cells after incubation with different concentrations of platinum complex. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Compound 6								
Untreated	100.0	4.4	0.567	0.025	100.0	1.9	0.556	0.011
-7.5	86.7	1.9	0.491	0.011	90.5	2.8	0.503	0.015
-6.5	79.2	2.7	0.449	0.015	87.4	9.4	0.485	0.052
-5.5	77.8	1.3	0.441	0.007	79.1	12.7	0.440	0.071
-5.2	70.5	1.2	0.400	0.007	93.0	7.5	0.517	0.042
-4.9	62.4	3.2	0.353	0.018	83.6	5.9	0.464	0.033
-4.6	49.1	4.9	0.278	0.028	76.3	9.7	0.424	0.054
-4.3	39.0	3.0	0.221	0.017	51.5	5.4	0.286	0.030
-4.1	23.7	1.6	0.134	0.009	42.3	3.9	0.235	0.022
-4.0	14.7	3.5	0.083	0.020	33.0	3.8	0.183	0.021
-3.7	4.0	0.3	0.023	0.002	7.2	0.5	0.040	0.003
Compound 7								
Untreated	100.0	2.3	0.471	0.011	100.0	2.7	0.460	0.012
-7.5	98.4	10.3	0.464	0.049	99.1	9.7	0.456	0.045
-6.5	89.5	7.0	0.422	0.033	89.7	3.7	0.413	0.017
-5.5	90.6	6.7	0.427	0.031	86.5	10.8	0.398	0.049
-5.2	76.1	7.3	0.359	0.034	88.2	0.8	0.406	0.003
-4.9	90.5	11.1	0.426	0.052	85.3	2.2	0.393	0.010
-4.6	82.5	11.6	0.398	0.055	88.8	2.2	0.409	0.010
-4.3	66.3	8.0	0.312	0.038	76.3	7.3	0.351	0.034
-4.1	53.5	5.5	0.252	0.026	49.9	0.8	0.230	0.004
-4.0	38.3	5.7	0.181	0.027	39.1	4.0	0.180	0.018
-3.7	5.8	0.2	0.027	0.001	17.3	3.0	0.080	0.014

SD, standard deviation; --, concentration was not used

A4 DNA platination

*DNA platination in HCT-8 and HCT-8 ox cells after incubation with oxaliplatin and **7** for 4 h [Platinum atoms/10^6 nucleotides].*

	Oxaliplatin		Compound 7	
	HCT-8	HCT-8ox	HCT-8	HCT-8ox
Median	36.33	15.06	7.82	4.88
IQR	21.78	6.53	5.32	2.06
n	6	6	6	6

n, number of experiments; IQR, interquartile range

A5 Repair of platinum-DNA adducts

Amount of platinum-DNA adducts in HCT-8 and HCT-8ox cells after 4 h of incubation with oxaliplatin and subsequent incubation with drug-free medium [Platinum atoms/10^6 nucleotides].

	HCT-8			HCT-8ox		
Time	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
0	28.64	22.22	6	29.06	12.64	6
2	30.47	14.57	6	31.19	10.09	6
4	35.31	25.22	6	28.36	10.05	6
6	36.75	22.35	6	31.82	8.59	6

n, number of experiments; IQR, interquartile range

Appendix B

Results of the investigation on oxaliplatin analogues with different leaving groups

B1 Determination of log P

log P values of the investigated platinum complexes.

Volume ratio ($V_{1\text{-octanol}}$:V_{water})	Pt_{water} [µM]	$Pt_{1\text{-octanol}}$ [µM]	log P
Compound 8			
1:1	9.25	0.48	-1.28
1:1	9.40	0.51	-1.27
1:2	8.96	0.51	-1.25
1:2	9.71	0.52	-1.27
2:1	8.47	0.36	-1.38
2:1	8.98	0.39	-1.37
Compound 9			
1:1	6.24	0.92	-0.83
1:1	6.79	0.86	-0.90
1:2	6.88	0.91	-0.88
1:2	6.97	0.98	-0.85
2:1	5.40	0.69	-0.89
2:1	5.51	0.86	-0.81
Compound 10			
1:1	4.67	0.12	-1.58
1:1	4.77	0.15	-1.51
1:2	4.15	0.12	-1.54
1:2	4.43	0.10	-1.63
2:1	4.63	0.13	-1.55
Compound 11			
1:1	8.06	0.05	-2.24
1:1	8.38	0.04	-2.28
1:2	7.92	0.03	-2.41
2:1	7.35	0.03	-2.41
2:1	7.53	0.03	-2.35
Compound 12			
1:1	7.70	0.02	-2.58
1:2	8.38	0.02	-2.71
1:2	8.54	0.02	-2.67
2:1	6.75	0.01	-2.67
2:1	7.57	0.01	-2.70

B2 Reactivity towards nucleotides

Reduction of the corrected peak area of the nucleotides dAMP and dGMP during incubation with platinum complex (n = 3, mean ± SD). The initial concentration of the nucleotides was set to 100 %.

Time [h]	dAMP [%]	SD [%]	dGMP [%]	SD [%]
Oxaliplatin				
0	100.0	5.3	100.0	2.6
12	97.6	4.9	94.4	1.6
24	83.5	1.7	79.6	2.0
48	74.8	5.8	60.7	2.8
72	66.5	3.7	46.6	0.3
Compound 8				
0	100.0	6.6	100.0	7.3
12	78.5	5.4	65.2	4.4
24	76.1	5.5	53.9	2.8
48	70.0	5.0	38.3	2.0
72	65.0	5.1	32.6	2.9
Compound 9				
0	100.0	6.4	100.0	7.0
12	105.4	7.8	101.9	7.5
24	95.8	8.9	99.5	9.1
48	91.5	7.8	85.7	7.4
72	88.3	7.0	80.2	6.6
Compound 10				
0	100.0	0.2	100.0	2.3
12	98.8	4.4	96.5	2.2
24	90.9	3.0	91.3	4.9
48	87.3	1.0	73.8	0.9
72	81.1	1.6	63.3	1.6
Compound 11				
0	100.0	2.1	100.0	0.9
12	102.6	3.0	105.5	5.0
24	98.7	5.9	99.24	4.8
48	108.2	2.4	109.9	2.1
72	97.1	1.1	98.5	2.4
Compound 12				
0	100.0	2.3	100.0	1.1
12	94.3	3.2	99.2	1.3
24	95.1	1.4	97.0	3.4
48	94.6	1.2	98.6	3.3
72	89.2	0.7	93.2	3.0

(Cont.) Reduction of the corrected peak area of the nucleotides dAMP and dGMP during incubation with platinum complex (n = 3, mean ± SD). The initial concentration of the nucleotides was set to 100 %.

Time [h]	dAMP [%]	SD [%]	dGMP [%]	SD [%]
Cisplatin				
0	100.0	3.2	100.0	0.9
12	80.1	2.1	52.2	2.6
24	60.6	3.7	25.6	3.1
48	43.1	4.6	10.6	2.3
72	42.2	9.6	7.4	3.1
Carboplatin				
0	100.0	2.1	100.0	3.4
12	95.7	5.8	97.8	5.8
24	97.0	6.2	97.5	5.8
48	94.6	2.1	94.1	2.8
72	92.8	2.9	84.4	4.7

SD, standard deviation

B3 Influx

Intracellular platinum concentration [µM] in HCT-8 and HCT-8ox cells after incubation with 100 µM platinum complex up to 120 min.

	HCT-8			HCT-8ox		
Time [min]	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
Oxaliplatin						
10	1.23	0.29	12	0.63	0.28	18
60	8.14	0.65	15	3.12	1.68	18
120	14.05	1.23	15	7.86	2.97	18
Compound 8						
10	1.81	0.10	6	0.84	0.67	6
60	13.58	2.72	6	5.90	2.38	6
120	24.68	3.66	6	12.35	3.89	6
Compound 9						
10	3.38	2.04	6	5.09	0.50	6
60	13.08	0.86	6	18.81	2.22	6
120	18.45	1.27	6	21.44	2.29	6
Compound 10						
10	0.74	0.60	6	0.47	0.54	6
60	2.69	0.40	6	1.70	0.59	6
120	4.02	2.01	6	2.27	0.92	6

(Cont.) Intracellular platinum concentration [µM] in HCT-8 and HCT-8ox cells after incubation with 100 µM platinum complex up to 120 min.

Time [min]	Median	IQR	n	Median	IQR	n
Compound 11						
10	0.22	0.05	6	0.57	0.24	6
60	0.70	0.03	6	0.60	0.33	6
120	1.26	0.63	6	1.30	0.32	6
Compound 12						
10	0.31	0.33	6	0.36	0.21	6
60	0.44	0.04	6	0.42	0.01	6
120	0.80	0.09	6	0.70	0.07	6
Cisplatin						
10	5.31	3.78	6	1.79	1.67	6
60	18.73	4.44	6	5.75	1.48	6
120	30.61	10.25	6	10.62	1.57	6
Carboplatin						
10	0.71	0.31	6	0.29	0.06	6
60	4.06	0.77	6	1.22	0.15	6
120	6.07	1.10	6	2.37	0.24	6

n, number of experiments; IQR, interquartile range

B4 Cytotoxicity

Survival of HCT-8 and HCT-8ox cells after incubation with different concentrations of platinum complex. The mean absorption determined in a representative experiment is indicated in the table (n = 3 - 11). The absorption of untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Oxaliplatin								
Untreated	100.0	2.8	0.891	0.025	100.0	9.4	0.449	0.042
-7.5	106.3	1.9	0.947	0.017	--	--	--	--
-7.0	99.6	7.7	0.887	0.069	128.4	14.3	0.577	0.064
-6.5	90.9	5.9	0.809	0.053	134.3	3.8	0.603	0.017
-6.0	53.5	4.3	0.477	0.038	123.0	1.9	0.552	0.009
-5.7	35.8	3.9	0.319	0.035	99.7	6.8	0.448	0.030
-5.3	33.3	2.3	0.288	0.020	89.0	5.9	0.400	0.027
-5.0	29.9	2.8	0.266	0.025	53.7	1.2	0.241	0.005
-4.5	25.0	0.7	0.223	0.006	36.2	1.0	0.163	0.005
-3.5	4.9	0.2	0.044	0.002	11.00	0.3	0.049	0.001
-3.0	--	--	--	--	10.8	0.3	0.048	0.001
Compound 8								
Untreated	100.0	9.6	0.519	0.050	100.0	20.6	0.352	0.073
-7.7	112.6	4.5	0.585	0.024	112.2	7.5	0.395	0.027
-7.2	107.5	3.7	0.558	0.019	108.9	10.3	0.383	0.036
-6.7	88.6	7.2	0.460	0.038	100.7	11.0	0.354	0.039
-6.2	52.2	1.5	0.271	0.008	107.2	5.1	0.377	0.018
-5.7	29.6	2.1	0.154	0.011	83.6	3.3	0.294	0.012
-5.2	22.9	0.7	0.119	0.004	52.4	1.7	0.184	0.006
-4.7	19.3	0.2	0.100	0.001	33.4	3.1	0.118	0.011
-4.2	14.6	0.3	0.076	0.002	25.1	1.7	0.088	0.006
-3.7	13.0	1.2	0.067	0.006	27.9	2.6	0.098	0.009
Compound 9								
Untreated	100.0	2.5	0.654	0.016	100.0	6.2	0.549	0.034
-7.7	105.3	6.3	0.688	0.041	93.6	6.7	0.514	0.037
-7.2	103.8	4.3	0.678	0.028	95.1	3.1	0.522	0.017
-6.7	100.7	3.7	0.658	0.024	83.3	4.0	0.457	0.022
-6.2	87.4	4.6	0.571	0.030	84.1	3.3	0.462	0.018
-5.7	58.6	2.7	0.383	0.018	78.5	5.6	0.431	0.031
-5.2	32.0	1.9	0.209	0.012	66.8	12.4	0.367	0.068
-4.7	28.2	3.0	0.184	0.020	37.4	7.7	0.205	0.042
-4.2	22.9	0.6	0.149	0.004	23.4	9.0	0.129	0.050
-3.7	5.5	0.3	0.036	0.002	11.1	1.0	0.061	0.006

(Cont.) Survival of HCT-8 and HCT-8ox cells after incubation with different concentrations of platinum complex. The mean absorption determined in a representative experiment is indicated in the table (n = 3 - 11). The absorption of untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Compound 10								
Untreated	100.0	0.6	1.101	0.007	100.0	7.7	0.956	0.073
-7.7	87.3	2.3	0.961	0.025	83.1	1.8	0.794	0.017
-7.2	86.9	1.8	0.957	0.020	85.7	4.2	0.819	0.041
-6.7	87.5	0.8	0.963	0.009	88.2	8.8	0.843	0.084
-6.2	73.7	7.4	0.811	0.082	90.6	5.6	0.866	0.053
-5.7	36.3	2.7	0.400	0.029	86.3	5.2	0.825	0.050
-5.2	30.4	3.3	0.335	0.037	64.3	3.6	0.614	0.035
-4.7	26.3	1.5	0.290	0.017	33.2	2.6	0.318	0.025
-4.2	17.0	0.7	0.187	0.008	25.9	2.1	0.248	0.021
-3.7	5.1	0.1	0.056	0.002	6.1	0.3	0.058	0.003
Compound 11								
Untreated	100.0	10.1	0.618	0.063	100.0	9.1	0.549	0.050
-4.9	85.5	9.1	0.528	0.056	79.5	3.6	0.436	0.020
-4.7	88.0	3.7	0.544	0.023	82.6	8.2	0.453	0.045
-4.4	83.9	2.5	0.518	0.016	90.6	0.8	0.497	0.004
-4.2	79.5	9.8	0.491	0.061	89.3	6.7	0.490	0.037
-3.9	59.0	3.2	0.365	0.020	79.0	5.6	0.433	0.031
-3.7	37.9	2.3	0.234	0.014	63.8	11.5	0.350	0.063
-3.4	31.8	1.8	0.197	0.011	46.5	4.1	0.255	0.023
-3.2	25.2	0.8	0.155	0.005	32.8	1.7	0.180	0.010
-2.9	9.6	0.3	0.059	0.002	17.0	0.9	0.093	0.005
Compound 12								
Untreated	100.0	4.1	0.563	0.023	100.0	0.1	0.412	0.001
-5.6	101.6	4.8	0.572	0.027	106.2	7.6	0.438	0.031
-4.4	97.1	5.3	0.547	0.030	108.3	6.8	0.446	0.028
-4.2	86.2	9.7	0.485	0.055	95.6	2.2	0.394	0.009
-4.0	77.2	9.3	0.434	0.053	94.4	7.0	0.389	0.029
-3.6	57.6	2.9	0.324	0.017	91.5	7.0	0.377	0.029
-3.4	34.8	3.5	0.196	0.020	72.9	10.1	0.301	0.042
-4.8	31.1	1.9	0.175	0.011	59.2	5.9	0.244	0.024
-3.2	27.8	1.8	0.157	0.010	48.5	1.9	0.200	0.008
-2.8	9.8	7.1	0.055	0.040	23.2	1.7	0.096	0.007

(Cont.) Survival of HCT-8 and HCT-8ox cells after incubation with different concentrations of platinum complex. The mean absorption determined in a representative experiment is indicated in the table (n = 3 - 11). The absorption of untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cisplatin								
Untreated	100.0	0.1	0.923	0.001	100.0	0.3	0.812	0.003
-7.5	100.3	7.0	0.926	0.064	85.4	3.9	0.695	0.032
-6.5	95.2	0.6	0.878	0.006	87.3	3.3	0.710	0.027
-5.5	76.0	2.8	0.701	0.026	78.5	2.3	0.639	0.018
-5.2	62.5	6.4	0.577	0.059	73.7	7.7	0.600	0.063
-4.9	47.3	2.7	0.436	0.025	56.9	1.6	0.463	0.013
-4.6	30.5	0.7	0.281	0.006	41.5	3.0	0.338	0.024
-4.3	11.4	0.4	0.105	0.004	31.5	1.1	0.256	0.009
-4.1	9.7	0.5	0.090	0.004	10.2	0.2	0.083	0.002
-4.0	9.2	1.3	0.085	0.012	8.6	0.2	0.070	0.002
-3.7	7.0	1.2	0.064	0.012	8.5	0.5	0.069	0.004
Carboplatin								
Untreated	100.0	14.3	0.625	0.089	100.0	13.7	0.549	0.075
-6.5	100.8	13.4	0.630	0.084	79.1	8.6	0.434	0.047
-5.0	102.3	3.5	0.639	0.022	78.6	4.3	0.431	0.024
-4.5	77.8	6.0	0.486	0.038	81.4	1.8	0.447	0.010
-4.3	57.4	2.9	0.359	0.018	73.5	1.8	0.404	0.010
-4.1	45.2	7.8	0.282	0.049	71.9	1.9	0.395	0.011
-4.0	--	--	--	--	64.0	3.8	0.351	0.021
-3.9	37.8	5.3	0.236	0.033	61.2	5.6	0.336	0.031
-3.7	23.9	3.3	0.149	0.020	47.3	7.7	0.260	0.042
-3.5	13.7	0.7	0.086	0.004	33.6	2.5	0.185	0.014
-3.0	9.7	0.3	0.060	0.002	12.4	0.8	0.068	0.005
-2.4	7.8	0.7	0.049	0.004	--	--	--	--

SD, standard deviation; **--,** concentration was not used

Appendix C

Results of the investigation on transport proteins

C1 Influx

Intracellular platinum concentration [µM] after incubation of HCT-8 and HCT-8ox cells with various extracellular concentrations of oxaliplatin for 2 h.

	HCT-8			HCT-8ox		
Extracellular concentration [µM]	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
20	2.93	0.61	6	1.30	0.24	6
100	16.12	1.94	6	5.77	0.76	6
500	79.52	3.99	6	30.92	5.55	6
1000	164.31	42.73	6	61.94	8.03	6
1500	265.33	16.89	6	88.46	4.53	6

n, number of experiments; IQR, interquartile range

Intracellular platinum concentration [µM] after incubation of HCT-8 and HCT-8ox cells with 100 µM oxaliplatin up to 4 h.

	HCT-8			HCT-8ox		
Time [min]	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
10	1.39	0.16	6	1.05	0.15	6
60	8.61	0.44	6	4.87	0.68	6
120	19.05	1.34	6	10.59	1.51	6
240	39.52	2.03	6	17.95	1.36	6

n, number of experiments; IQR, interquartile range

Intracellular platinum concentration [µM] in HCT-8 and HCT-8ox cells after incubation with 100 µM oxaliplatin and after coincubation of 100 µM oxaliplatin and 50 µM or 100 µM copper sulfate

	HCT-8			HCT-8ox		
Treatment	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
Oxaliplatin	12.94	7.69	12	6.98	2.12	9
Coincubation, 100 µM copper	14.87	2.30	7	5.21	0.66	6
Coincubation, 50 µM copper	13.18	5.97	9	6.15	0.71	6

n, number of experiments; IQR, interquartile range

Intracellular platinum concentration [µM] in HCT-8 and HCT-8ox cells after incubation with 100 µM oxaliplatin and after coincubation of 100 µM oxaliplatin and 10 mM, 100 µM or 1 µM.

	HCT-8			HCT-8ox		
Treatment	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
Oxaliplatin	16.49	4.39	6	6.33	0.46	6
Coincubation, 10 mM TEA	10.87	1.56	6	4.92	0.84	6
Coincubation, 100 µM TEA	11.49	2.19	6	5.38	1.20	6
Coincubation, 1 µM TEA	13.01	3.58	6	5.41	1.65	6

n, number of experiments; IQR, interquartile range

Intracellular platinum concentration [µM] in HCT-8 and HCT-8ox cells after incubation with 100 µM oxaliplatin and after pre- or coincubation of 100 µM oxaliplatin and 1 mM atropine.

	HCT-8			HCT-8ox		
Treatment	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
Oxaliplatin	23.00	4.32	9	7.89	2.35	9
Coincubation, 1 mM atropine	16.58	4.54	6	7.89	1.40	6
Preincubation, 1 h, 1 mM atropine	18.72	5.52	9	6.73	1.05	8

n, number of experiments; IQR, interquartile range

Intracellular platinum concentration [µM] in HCT-8 and HCT-8ox cells after incubation with 100 µM oxaliplatin and after pre- or coincubation of 100 µM oxaliplatin and 1.5 mM cimetidine.

	HCT-8			HCT-8ox		
Treatment	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
Oxaliplatin	19.76	2.70	9	10.59	1.51	6
Coincubation, 1.5 mM cimetidine	14.58	2.42	9	7.90	2.55	9
Preincubation, 1 h, 1.5 mM cimetidine	15.49	1.92	12	6.80	2.32	10

n, number of experiments; IQR, interquartile range

Intracellular platinum concentration [µM] in HCT-8 and HCT-8ox cells after incubation with 100 µM oxaliplatin and after coincubation of 100 µM oxaliplatin and 1 µM or 0.1 µM decycnium-22.

	HCT-8			HCT-8ox		
Treatment	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
Oxaliplatin	16.50	1.48	9	6.18	2.28	9
Coincubation, 1 µM decynium-22	15.53	1.86	9	5.81	1.11	8
Coincubation, 0.1 µM decynium-22	15.98	3.24	9	7.00	2.37	8

n, number of experiments; IQR, interquartile range

C2 Cytotoxicity

Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after coincubation of oxaliplatin and 100 µM or 50 µM copper sulfate. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Oxaliplatin								
Untreated	100.0	5.3	0.534	0.028	100.0	9.1	0.528	0.048
-7.5	100.9	9.1	0.539	0.049	--	--	--	--
-7.0	99.3	4.7	0.530	0.025	99.7	4.2	0.527	0.022
-6.5	93.5	9.3	0.499	0.049	98.2	7.7	0.518	0.041
-6.0	53.3	3.2	0.285	0.017	107.3	4.5	0.567	0.024
-5.5	34.5	0.1	0.184	0.001	102.3	7.8	0.540	0.041
-5.0	31.2	1.7	0.167	0.009	69.9	12.3	0.369	0.065
-4.5	26.7	0.4	0.143	0.002	39.6	2.8	0.209	0.015
-4.0	11.6	1.0	0.062	0.006	26.6	2.9	0.140	0.015
-3.5	10.9	0.4	0.058	0.002	10.4	1.2	0.055	0.006
	--	--	--	--	9.8	1.2	0.052	0.006
Coincubation, 100 µM copper sulfate								
Untreated	76.3	2.1	0.407	0.011	70.3	6.1	0.371	0.032
-7.5	82.8	7.6	0.442	0.041	--	--	--	--
-7.0	83.8	11.1	0.447	0.060	66.2	14.2	0.349	0.075
-6.5	67.6	11.3	0.361	0.060	64.4	10.3	0.340	0.054
-6.0	32.4	4.8	0.173	0.026	65.6	10.9	0.346	0.058
-5.5	24.2	1.4	0.129	0.007	59.5	7.8	0.314	0.041
-5.0	23.3	1.1	0.125	0.006	35.4	7.9	0.187	0.042
-4.5	15.0	3.0	0.080	0.016	21.5	4.1	0.113	0.022
-4.0	7.9	0.9	0.042	0.005	12.8	2.5	0.068	0.013
-3.5	7.6	0.7	0.041	0.004	8.7	0.2	0.046	0.001
-3.0	--	--	--	--	7.6	0.2	0.040	0.001
Coincubation, 50 µM copper sulfate								
Untreated	80.5	3.2	0.430	0.017	90.1	2.4	0.476	0.013
-7.5	93.5	13.6	0.499	0.073	--	--	--	--
-7.0	84.8	2.6	0.453	0.014	90.7	4.6	0.479	0.024
-6.5	74.9	8.1	0.400	0.043	92.3	2.4	0.487	0.013
-6.0	40.1	1.4	0.214	0.008	88.6	1.7	0.468	0.009
-5.5	33.1	5.4	0.177	0.029	89.5	7.7	0.472	0.041
-5.0	27.2	0.9	0.145	0.005	56.2	6.7	0.297	0.035
-4.5	21.8	1.7	0.117	0.009	34.5	2.4	0.182	0.013
-4.0	8.5	0.2	0.045	0.001	23.9	3.7	0.126	0.019
-3.5	8.5	0.9	0.045	0.005	10.1	0.3	0.053	0.002
-3.0	--	--	--	--	9.0	0.3	0.048	0.002

SD, standard deviation; **--,** concentration was not used

Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after coincubation of oxaliplatin and 10 mM, 10 mM or 10 µM TEA. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Oxaliplatin								
Untreated	100.0	1.9	0.501	0.009	100.0	12.8	0.574	0.073
-7.5	79.1	3.7	0.396	0.019	--	--	--	--
-7.0	82.9	1.8	0.415	0.009	88.7	10.5	0.509	0.060
-6.5	73.4	3.1	0.368	0.016	87.2	3.8	0.500	0.022
-6.0	44.0	0.8	0.221	0.004	92.1	6.4	0.529	0.037
-5.5	27.7	3.0	0.139	0.015	87.9	4.7	0.505	0.027
-5.0	22.3	0.4	0.112	0.002	65.9	5.8	0.378	0.033
-4.5	18.0	0.7	0.090	0.004	36.5	1.1	0.210	0.007
-4.0	6.5	0.3	0.033	0.002	25.5	1.0	0.146	0.006
-3.5	5.5	0.1	0.028	0.001	6.3	0.3	0.036	0.002
-3.0	--	--	--	--	6.9	0.1	0.039	0.001
Coincubation, 10 mM TEA								
Untreated	79.8	2.5	0.400	0.012	77.0	8.6	0.442	0.050
-7.5	90.7	13.5	0.454	0.068	--	--	--	--
-7.0	87.8	3.5	0.440	0.018	85.6	10.3	0.491	0.059
-6.5	67.6	5.5	0.339	0.027	78.9	4.2	0.453	0.024
-6.0	41.4	3.0	0.207	0.015	76.1	6.8	0.437	0.039
-5.5	24.7	0.6	0.124	0.003	70.4	12.6	0.404	0.073
-5.0	21.8	1.1	0.109	0.005	58.1	6.2	0.333	0.036
-4.5	20.6	0.8	0.103	0.004	31.5	1.8	0.181	0.010
-4.0	8.2	0.5	0.041	0.003	23.0	0.8	0.132	0.005
-3.5	6.9	0.9	0.035	0.005	6.9	0.2	0.039	0.001
-3.0	--	--	--	--	6.6	0.3	0.038	0.002
Coincubation, 1 mM TEA								
Untreated	80.1	8.8	0.401	0.044	90.5	4.1	0.520	0.024
-7.5	92.4	1.6	0.463	0.008	--	--	--	--
-7.0	81.3	5.5	0.407	0.028	86.3	0.3	0.495	0.002
-6.5	75.8	2.5	0.380	0.013	85.9	13.7	0.493	0.078
-6.0	45.3	3.2	0.227	0.016	82.5	5.3	0.473	0.030
-5.5	23.8	2.9	0.119	0.014	83.6	9.1	0.480	0.052
-5.0	23.3	2.1	0.117	0.011	63.7	4.2	0.366	0.024
-4.5	19.6	1.1	0.098	0.006	33.3	1.8	0.191	0.010
-4.0	8.0	0.2	0.040	0.001	22.3	0.8	0.128	0.004
-3.5	7.6	0.2	0.038	0.001	6.9	0.1	0.040	0.001
-3.0	--	--	--	--	7.5	0.0	0.043	0.000

(Cont.) Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after coincubation of oxaliplatin and 10 mM, 10 mM or 10 µM TEA. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Coincubation, 10 µM TEA								
Untreated	83.2	4.0	0.417	0.020	78.7	11.5	0.452	0.066
-7.5	108.4	6.5	0.543	0.032	--	--	--	--
-7.0	103.1	5.1	0.517	0.026	105.6	2.7	0.606	0.015
-6.5	91.4	2.3	0.458	0.012	99.7	4.5	0.572	0.026
-6.0	47.9	5.0	0.240	0.025	98.1	2.4	0.563	0.014
-5.5	26.4	0.8	0.132	0.004	93.1	3.3	0.534	0.019
-5.0	22.7	1.6	0.114	0.008	72.6	2.2	0.417	0.013
-4.5	20.3	1.1	0.102	0.006	32.5	2.8	0.186	0.016
-4.0	7.9	0.1	0.039	0.001	22.8	1.4	0.131	0.008
-3.5	6.1	0.7	0.030	0.004	6.8	0.3	0.039	0.002
-3.0	--	--	--	--	6.6	0.3	0.038	0.002

SD, standard deviation, --, Concentration was not used.

Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after preincubation with 1 mM atropine for 1 h and subsequent incubation with oxaliplatin alone, as well as after coincubation of oxaliplatin and 1 mM or 100 µM atropine. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Oxaliplatin								
Untreated	100.0	7.9	0.469	0.037	100.0	9.1	0.478	0.043
-7.5	112.9	2.8	0.529	0.013	--	--	--	--
-7.0	112.9	4.6	0.529	0.022	109.6	4.1	0.524	0.019
-6.5	100.1	16.4	0.469	0.077	116.7	9.8	0.558	0.047
-6.0	51.7	6.4	0.243	0.030	113.0	4.8	0.540	0.023
-5.5	30.9	1.2	0.145	0.006	112.6	4.6	0.538	0.022
-5.0	30.8	1.7	0.145	0.008	87.3	5.5	0.417	0.026
-4.5	27.1	0.4	0.127	0.002	44.1	1.1	0.211	0.005
-4.0	9.4	0.2	0.044	0.001	31.4	1.3	0.150	0.006
-3.5	5.8	0.2	0.027	0.001	8.4	0.2	0.040	0.001
-3.0	--	--	--	--	8.6	0.1	0.041	0.001

(Cont.) Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after coincubation of oxaliplatin and 10 mM, 10 mM or 10 µM TEA. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Preincubation, 1 h, 1 mM atropine								
Untreated	85.8	5.0	0.402	0.023	97.4	4.2	0.466	0.020
-7.5	94.6	12.7	0.444	0.060	--	--	--	--
-7.0	95.7	7.4	0.449	0.035	103.6	6.7	0.495	0.032
-6.5	84.8	5.0	0.398	0.024	113.1	8.0	0.541	0.038
-6.0	47.0	5.1	0.220	0.024	99.2	5.0	0.474	0.024
-5.5	29.1	2.1	0.137	0.010	107.2	0.9	0.512	0.005
-5.0	28.6	2.0	0.134	0.009	81.9	7.9	0.391	0.038
-4.5	23.8	1.5	0.112	0.007	39.7	4.6	0.190	0.022
-4.0	8.2	0.1	0.039	0.001	30.1	2.6	0.144	0.013
-3.5	5.6	0.3	0.026	0.002	6.7	0.4	0.032	0.002
-3.0	--	--	--	--	6.6	0.1	0.032	0.001
Coincubation, 1 mM atropine								
Untreated	46.1	7.5	0.216	0.035	53.9	6.6	0.258	0.031
-7.5	63.3	0.5	0.297	0.002	--	--	--	--
-7.0	66.4	4.0	0.311	0.019	68.3	3.9	0.327	0.019
-6.5	59.8	1.0	0.280	0.005	64.6	3.6	0.309	0.017
-6.0	56.6	2.3	0.265	0.011	71.4	4.3	0.341	0.021
-5.5	38.1	0.6	0.179	0.003	64.4	6.4	0.308	0.031
-5.0	31.4	1.0	0.147	0.005	65.8	2.7	0.315	0.013
-4.5	22.5	0.5	0.106	0.003	46.6	5.0	0.223	0.024
-4.0	9.0	0.7	0.042	0.003	29.8	3.0	0.143	0.015
-3.5	5.5	0.2	0.026	0.001	6.9	0.2	0.033	0.001
-3.0	--	--	--	--	7.7	0.1	0.037	0.001
Coincubation, 100 µM atropine								
Untreated	103.9	7.3	0.487	0.034	109.6	5.9	0.524	0.028
-7.5	111.2	8.6	0.521	0.041	--	--	--	--
-7.0	123.2	12.4	0.578	0.058	130.3	3.2	0.623	0.015
-6.5	117.2	4.2	0.550	0.020	123.6	5.1	0.591	0.024
-6.0	59.3	4.4	0.278	0.021	117.6	7.8	0.562	0.038
-5.5	36.5	2.4	0.171	0.011	129.8	3.1	0.621	0.015
-5.0	31.33	5.1	0.147	0.024	103.0	4.9	0.492	0.023
-4.5	31.1	3.7	0.146	0.017	52.8	5.4	0.252	0.026
-4.0	10.2	0.2	0.048	0.001	34.2	2.0	0.163	0.010
-3.5	5.1	0.6	0.024	0.003	6.9	0.4	0.033	0.002
-3.0	--	--	--	--	7.3	0.0	0.035	0.000

SD, standard deviation, --, Concentration was not used.

Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after preincubation with 1.5 mM cimetidine for 6 h and subsequent incubation with oxaliplatin alone, as well as after coincubation of oxaliplatin and 1.5 mM or 0.15 mM cimetidine. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Oxaliplatin								
Untreated	100.0	2.5	0.505	0.012	100.0	12.2	0.540	0.066
-7.5	96.1	1.6	0.476	0.008	--	--	--	--
-7.0	94.5	3.5	0.483	0.018	94.9	6.5	0.513	0.035
-6.5	81.7	1.8	0.403	0.009	98.6	7.2	0.532	0.039
-6.0	37.8	2.0	0.200	0.010	98.6	2.5	0.533	0.014
-5.5	27.5	0.7	0.143	0.004	95.8	5.6	0.517	0.030
-5.0	24.5	1.8	0.134	0.009	58.5	4.0	0.316	0.027
-4.5	19.9	0.8	0.104	0.004	29.9	2.4	0.162	0.013
-4.0	5.7	0.2	0.029	0.001	21.0	2.1	0.114	0.012
-3.5	4.8	0.2	0.024	0.001	5.6	0.4	0.030	0.002
-3.0	--	--	--	--	5.4	0.2	0.029	0.001
Preincubation, 6 h, 1.5 mM cimetidine								
Untreated	100.0	8.5	0.505	0.043	94.7	1.4	0.511	0.007
-7.5	91.2	5.7	0.460	0.029	--	--	--	--
-7.0	95.8	8.8	0.484	0.045	94.6	7.2	0.511	0.039
-6.5	87.0	9.2	0.439	0.046	88.7	2.5	0.479	0.014
-6.0	67.9	15.6	0.343	0.079	94.3	10.7	0.509	0.058
-5.5	30.4	9.9	0.154	0.050	110.2	11.4	0.595	0.062
-5.0	25.3	1.8	0.128	0.009	95.1	8.0	0.514	0.043
-4.5	22.0	1.4	0.111	0.007	53.1	10.5	0.287	0.057
-4.0	16.2	2.5	0.082	0.013	29.6	0.8	0.160	0.005
-3.5	4.7	0.1	0.024	0.001	11.9	3.3	0.064	0.018
-3.0	--	--	--	--	5.9	0.2	0.032	0.001
Coincubation, 1.5 mM cimetidine								
Untreated	91.0	10.0	0.460	0.050	87.4	6.1	0.472	0.033
-6.5	101.5	2.1	0.513	0.011	92.2	5.9	0.498	0.032
-6.0	103.6	1.5	0.523	0.007	106.0	3.6	0.573	0.019
-5.5	97.5	8.9	0.492	0.045	98.0	5.7	0.529	0.031
-5.0	77.1	7.4	0.389	0.037	110.6	12.9	0.597	0.069
-4.5	39.9	1.5	0.201	0.008	103.8	2.1	0.560	0.012
-4.0	26.5	1.4	0.134	0.007	82.3	2.5	0.445	0.013
-3.5	23.2	3.2	0.117	0.016	53.2	5.9	0.287	0.032
-3.0	4.5	0.1	0.023	0.001	21.0	1.9	0.114	0.010
-2.5	3.8	0.0	0.019	0.000	3.3	0.4	0.018	0.002

(Cont.) Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after preincubation with 1.5 mM cimetidine for 6 h and subsequent incubation with oxaliplatin alone, as well as after coincubation of oxaliplatin and 1.5 mM or 0.15 mM cimetidine. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Coincubation, 0.15 mM cimetidine								
Untreated	73.7	2.5	0.372	0.013	92.0	5.7	0.497	0.031
-7.5	82.3	6.7	0.416	0.034	--	--	--	--
-7.0	82.6	9.0	0.417	0.045	94.0	11.2	0.508	0.060
-6.5	78.0	3.9	0.394	0.020	97.3	18.0	0.525	0.097
-6.0	53.9	11.8	0.272	0.059	84.8	4.1	0.458	0.022
-5.5	28.2	1.2	0.142	0.006	85.1	3.9	0.459	0.021
-5.0	23.0	1.9	0.116	0.010	78.3	9.6	0.423	0.052
-4.5	18.5	0.6	0.094	0.003	49.0	2.2	0.264	0.012
-4.0	10.9	0.7	0.055	0.003	25.2	2.4	0.136	0.013
-3.5	4.5	0.2	0.023	0.001	13.1	1.8	0.071	0.010
-3.0	--	--	--	--	5.6	0.5	0.030	0.003

SD, standard deviation; --, concentration was not used

Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after coincubation of oxaliplatin and 1 µM or 0.1 µM decynium-22. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Oxaliplatin								
Untreated	100.0	1.6	0.633	0.010	100.0	14.9	1.095	0.164
-7.5	101.1	1.7	0.640	0.011	--	--	--	--
-7.0	88.3	8.7	0.559	0.055	100.7	15.7	1.103	0.172
-6.5	68.1	3.0	0.431	0.019	93.4	10.2	1.022	0.112
-6.0	37.1	1.4	0.235	0.009	85.6	16.0	0.938	0.176
-5.5	29.0	1.1	0.183	0.007	98.7	5.4	1.081	0.059
-5.0	27.1	1.4	0.172	0.009	52.5	4.4	0.575	0.048
-4.5	15.6	0.7	0.099	0.005	29.1	2.5	0.318	0.027
-4.0	8.3	0.2	0.053	0.002	18.9	0.6	0.207	0.007
-3.5	7.8	0.2	0.050	0.002	6.6	0.1	0.073	0.002
	--	--	--	--	5.5	0.2	0.060	0.003
Coincubation, 1 µM decynium-22								
Untreated	60.0	3.0	0.380	0.019	49.3	1.1	0.540	0.012
-7.5	59.1	3.8	0.374	0.024	--	--	--	--
-7.0	58.6	3.0	0.371	0.019	50.3	9.8	0.550	0.108
-6.5	48.3	4.5	0.306	0.028	51.7	6.3	0.566	0.069
-6.0	23.4	1.5	0.148	0.010	43.9	2.5	0.481	0.027
-5.5	18.0	0.2	0.114	0.002	55.5	3.3	0.607	0.036
-5.0	18.3	1.1	0.116	0.007	35.2	3.4	0.385	0.037
-4.5	14.4	0.7	0.091	0.005	21.8	1.2	0.239	0.013
-4.0	8.3	0.6	0.052	0.004	16.2	1.6	0.178	0.018
-3.5	7.6	0.2	0.048	0.002	5.6	0.3	0.061	0.004
-3.0	--	--	--	--	5.2	0.1	0.057	0.001
Coincubation, 0.1 µM decynium-22								
Untreated	61.2	3.3	0.388	0.021	68.9	4.3	0.754	0.047
-7.5	63.8	1.0	0.404	0.006	--	--	--	--
-7.0	67.9	9.7	0.430	0.062	73.6	5.8	0.806	0.063
-6.5	52.6	4.6	0.333	0.029	71.9	6.8	0.787	0.074
-6.0	20.5	0.9	0.130	0.006	71.9	8.1	0.787	0.089
-5.5	17.9	0.6	0.113	0.004	64.6	5.5	0.707	0.061
-5.0	15.3	0.1	0.097	0.001	39.1	1.2	0.429	0.014
-4.5	11.8	0.4	0.075	0.002	19.1	1.5	0.209	0.016
-4.0	7.8	0.2	0.050	0.001	15.3	0.8	0.167	0.009
-3.5	8.2	0.3	0.052	0.002	5.1	0.1	0.057	0.002
-3.0	--	--	--	--	5.9	0.4	0.064	0.004

SD, standard deviation; --, concentration was not used

C3 Gene expression profile

Results of the qRT-PCR analysis. Ct values (cycle threshold; mean, SD, n), quantities (Q_{GOI}) calculated based on the Ct values, normalisation factors (NF) calculated based on the Ct values of the housekeeping genes, the normalized expression (GOI_{nE}) and the rescaled normalized expression (GOI_{rnE}) obtained by setting GOI_{nE} in untreated HCT-8 cells to 1 are presented in the table.

	Treatment	Mean Ct	SD Ct	n	Q_{GOI}	NF	GOI_{nE}	GOI_{rnE}
hCTR1				**HCT-8**				
	Untreated	22.955	0.047	2	0.833	0.620	1.343	1.000
	2 h + 24 h	22.690	0.062	2	1.000	1.000	1.000	0.745
	24 h + 24 h	24.091	0.032	2	0.379	0.673	0.563	0.419
	24 h + 0 h	26.128	0.227	2	0.092	0.335	0.275	0.205
				HCT-8ox				
	Untreated	24.513	0.067	2	0.283	0.449	0.630	0.469
	2 h + 24 h	23.724	0.199	2	0.488	0.581	0.840	0.626
	24 h + 24 h	23.920	0.136	2	0.426	0.454	0.939	0.699
	24 h + 0 h	23.422	0.071	2	0.602	0.748	0.805	0.600
hOCT1				**HCT-8**				
	Untreated	34.058	0.443	2	0.081	0.185	0.437	1.000
	2 h + 24 h	34.566	0.154	2	0.057	0.382	0.149	0.340
	24 h + 24 h	33.416	0.093	2	0.126	0.257	0.491	1.123
	24 h + 0 h	33.155	0.697	2	0.151	0.128	1.179	2.763
	24 h + 0 h	30.857	0.082	2	0.744	0.602	1.236	
				HCT-8ox				
	Untreated	34.211	0.267	2	0.073	0.148	0.490	1.603
	Untreated	31.096	0.590	2	0.630	0.629	1.001	
	2 h + 24 h	34.553	0.464	2	0.057	0.222	0.258	0.925
	2 h + 24 h	31.095	0.246	2	0.630	0.997	0.632	
	24 h + 24 h	33.925	1.015	2	0.089	0.185	0.478	1.192
	24 h + 24 h	31.528	1.320	2	0.467	0.822	0.568	
	24 h + 0 h	33.369	0.188	2	0.130	0.245	0.531	1.215

(Cont.) Results of the qRT-PCR analysis. Ct values (cycle threshold; mean, SD, n), quantities (Q_{GOI}) calculated based on the Ct values, normalisation factors (NF) calculated based on the Ct values of the housekeeping genes, the normalized expression (GOI_{nE}) and the rescaled normalized expression (GOI_{rnE}) obtained by setting GOI_{nE} in untreated HCT-8 cells to 1 are presented in the table.

	Treatment	Mean Ct	SD Ct	n	Q_{GOI}	NF	GOI_{nE}	GOI_{rnE}
				HCT-8				
hOCT2	Untreated	35.331	0.260	2	0.068	0.150	0.454	1.000
	Untreated	32.838	1.041	2	0.383	0.654	0.585	
	2 h + 24 h	36.821	0.174	2	0.024	0.242	0.100	0.369
	2 h + 24 h	32.925	0.494	2	0.361	1.000	0.361	
	24 h + 24 h	36.853	0.361	2	0.024	0.163	0.146	0.282
	24 h + 0 h	37.963	0.651	2	0.011	0.081	0.135	0.419
	24 h + 0 h	34.385	2.012	2	0.131	0.381	0.344	
				HCT-8ox				
	Untreated	36.437	1.396	2	0.032	0.094	0.337	0.640
	Untreated	34.413	0.296	2	0.129	0.398	0.323	
	2 h + 24 h	37.045	0.796	2	0.021	0.141	0.148	0.412
	2 h + 24 h	33.831	0.058	2	0.192	0.631	0.305	
	24 h + 24 h	35.112	0.280	2	0.079	0.117	0.674	2.208
	24 h + 24 h	31.453	0.231	2	1.000	0.521	1.921	
	24 h + 0 h	36.796	0.586	2	0.025	0.155	0.159	0.348
	24 h + 0 h	34.077	2.155	2	0.162	0.800	0.203	
				HCT-8				
hOCT3	Untreated	29.873	0.085	2	0.608	0.655	0.928	1.000
	2 h + 24 h	29.156	0.192	2	1.000	0.964	1.037	1.118
	24 h + 24 h	29.256	0.072	2	0.933	0.910	1.025	1.104
	24 h + 0 h	31.683	0.281	2	0.173	0.397	0.437	0.471
				HCT-8ox				
	Untreated	31.697	0.217	2	0.172	0.410	0.419	0.451
	2 h + 24 h	31.074	0.263	2	0.265	0.599	0.442	0.476
	24 h + 24 h	30.165	0.210	2	0.497	0.511	0.973	1.048
	24 h + 0 h	30.738	0.053	2	0.334	0.815	0.410	0.442

SD, standard deviation, n, number of experiments

Appendix D

Results of the investigation on interaction between oxaliplatin and cimetidine

D1 Cytotoxicity

Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after preincubation with 1.5 mM cimetidine for 12 h and subsequent incubation with oxaliplatin alone, as well as after coincubation of oxaliplatin and 1.5 mM or 0.15 mM cimetidine. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Oxaliplatin								
Untreated	100.0	2.5	0.505	0.012	100.0	12.2	0.540	0.066
-7.5	96.1	1.6	0.485	0.008	--	--	--	--
-7.0	94.5	3.5	0.477	0.018	94.9	6.5	0.513	0.035
-6.5	81.7	1.8	0.413	0.009	98.5	7.2	0.532	0.039
-6.0	37.8	2.0	0.191	0.010	98.6	2.5	0.533	0.014
-5.5	27.5	0.7	0.149	0.004	95.7	5.6	0.517	0.030
-5.0	24.5	1.8	0.124	0.009	58.4	4.9	0.316	0.027
-4.5	19.9	0.8	0.101	0.004	29.9	2.4	0.162	0.013
-4.0	5.7	0.2	0.029	0.001	21.0	2.1	0.114	0.012
-3.5	4.8	0.2	0.024	0.001	5.6	0.4	0.030	0.002
-3.0	--	--	--	--	5.4	0.2	0.029	0.001
Preincubation, 6 h, 1.5 mM cimetidine								
Untreated	100.0	8.5	0.505	0.043	94.7	1.4	0.511	0.007
-7.5	91.2	5.7	0.460	0.029	--	--	--	--
-7.0	95.8	8.8	0.484	0.045	94.6	7.2	0.511	0.039
-6.5	87.0	9.2	0.439	0.046	88.7	2.5	0.479	0.014
-6.0	67.9	15.6	0.343	0.079	94.3	10.7	0.509	0.058
-5.5	30.4	9.9	0.154	0.050	110.2	11.4	0.595	0.062
-5.0	25.3	1.8	0.128	0.009	95.1	8.0	0.514	0.043
-4.5	22.0	1.4	0.111	0.007	53.1	10.5	0.287	0.057
-4.0	16.2	2.5	0.082	0.013	29.6	0.8	0.160	0.005
-3.5	4.7	0.1	0.024	0.001	11.9	3.3	0.064	0.018
-3.0	--	--	--	--	5.9	0.2	0.032	0.001

(Cont.) Survival of HCT-8 and HCT-8ox cells after incubation with oxaliplatin alone and after preincubation with 1.5 mM cimetidine for 12 h and subsequent incubation with oxaliplatin alone, as well as after coincubation of oxaliplatin and 1.5 mM or 0.15 mM cimetidine. The mean absorption determined in a representative experiment is indicated in the table (n = 3). The absorption of oxaliplatin-untreated cells was set to 100 %.

	HCT-8				HCT-8ox			
	Absorption [%]		Absorption [units]		Absorption [%]		Absorption [units]	
log [conc.]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Preincubation, 12 h, 1.5 mM cimetidine								
Untreated	100.0	5.4	0.369	0.020	100.0	1.2	0.543	0.006
-7.5	93.8	1.2	0.346	0.004	--	--	--	--
-7.0	87.4	1.7	0.323	0.006	94.8	0.5	0.514	0.003
-6.5	81.4	3.6	0.300	0.013	94.6	5.4	0.513	0.029
-6.0	42.6	1.8	0.157	0.007	94.2	3.1	0.511	0.017
-5.5	28.0	2.4	0.103	0.009	87.3	1.7	0.474	0.009
-5.0	24.0	2.6	0.089	0.010	66.2	4.5	0.359	0.025
-4.5	18.5	1.3	0.068	0.005	32.2	4.1	0.175	0.022
-4.0	8.1	0.3	0.030	0.001	21.3	2.2	0.115	0.012
-3.5	5.4	0.3	0.020	0.001	4.7	0.6	0.025	0.003
-3.0	--	--	--	--	4.7	0.5	0.025	0.003
Coincubation, 1.5 mM cimetidine								
Untreated	91.0	10.0	0.460	0.050	87.4	6.1	0.472	0.033
-6.5	101.5	2.1	0.513	0.011	92.2	5.9	0.498	0.032
-6.0	103.6	1.5	0.523	0.007	106.0	3.6	0.573	0.019
-5.5	97.5	8.9	0.492	0.045	98.0	5.7	0.529	0.031
-5.0	77.1	7.4	0.389	0.037	110.6	12.9	0.597	0.069
-4.5	39.9	1.5	0.201	0.008	103.8	2.1	0.560	0.012
-4.0	26.5	1.4	0.134	0.007	82.3	2.5	0.445	0.013
-3.5	23.2	3.2	0.117	0.016	53.2	5.9	0.287	0.032
-3.0	4.5	0.1	0.023	0.001	21.0	1.9	0.114	0.010
-2.5	3.8	0.0	0.019	0.000	3.3	0.4	0.018	0.002

SD, standard deviation; --, concentration was not used

D2 Influx

Protein concentration in HCT-8 and HCT-8ox cells [µg protein/mL cell suspension]. The mean protein concentration of each day (n = 6), the within-day precision (SD [%], within-day) and the between-day precision (SD [%], between-day) are presented in the table.

Number of cells	HCT-8			HCT-8ox		
	Mean	SD [%], within-day	SD [%], between-day	Mean	SD [%], within-day	SD [%], between-day
1,000,000	233.7	12.2		488.6	7.2	
	263.0	9.6		548.8	11.5	
	237.3	9.6		482.7	6.1	
	313.7	13.4		470.4	7.9	
	231.1	7.2		511.9	4.5	
	222.5	14.3	13.6	473.5	5.9	6.0
2,000,000	544.4	6.0		1106.6	7.9	
	616.1	11.2		1004.3	10.1	
	517.0	4.7		894.3	8.8	
	662.2	9.8		990.6	8.1	
	522.3	6.4		1016.9	4.6	
	548.0	9.7	10.2	995.1	9.1	8.1
3,000,000	791.8	10.6		1438.3	7.3	
	927.6	10.9		1657.8	10.4	
	837.6	4.9		1503.3	5.2	
	891.0	13.7		1454.0	10.4	
	827.8	11.0		1531.4	10.2	
	778.2	8.9	6.8	1554.8	11.5	5.2
4,000,000	1167.8	4.5		2005.6	11.2	
	1071.5	9.1		1760.1	13.3	
	1093.5	3.5		1988.0	6.6	
	1267.8	13.2		2057.4	3.4	
	1145.5	6.9		2074.7	7.1	
	1165.7	7.1	6.0	1936.3	5.1	5.8

SD, standard deviation

Intracellular platinum concentration [ng/mg protein] in HCT-8 and HCT-8ox cells after incubation with 20 µM oxaliplatin up to 24 h, after coincubation with 20 µM oxaliplatin and 1.5 µM cimetidine and after preincubation with 20 µM oxaliplatin for 12 h and subsequent incubation with 20 µM oxaliplatin alone.

	HCT-8			HCT-8ox		
Time [h]	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**
Oxaliplatin						
2	6.26	2.69	9	2.16	0.84	9
4	11.26	1.61	9	5.30	1.10	9
6	15.22	2.86	9	5.71	3.36	9
8	25.54	8.74	9	11.01	8.72	9
10	30.26	5.52	9	13.81	3.21	9
12	27.71	8.33	9	11.66	5.81	9
24	33.50	2.22	9	18.34	1.15	9
Preincubation, 12 h, 1.5 mM cimetidine						
2	5.70	2.84	9	2.68	1.63	9
4	10.44	2.58	9	4.91	1.84	9
6	14.03	3.52	9	6.87	1.03	9
8	20.50	6.09	9	10.23	4.65	9
10	31.29	1.18	9	14.34	0.91	9
12	23.88	0.48	9	12.50	0.34	9
24	29.02	7.50	9	15.15	4.01	9
Coincubation, 1.5 mM cimetidine						
2	2.98	1.23	6	2.09	0.28	6
4	5.10	1.06	6	2.72	0.38	6
6	5.69	0.97	6	4.29	1.47	6
8	6.42	2.38	6	3.80	2.23	6
10	6.87	2.34	3	5.48	2.51	3
12	6.71	1.31	3	5.78	0.86	4
24	5.44	0.71	6	4.06	0.58	6

n, number of experiments; IQR, interquartile range

Publikationen, Kongressbeiträge

Wissenschaftliche Originalarbeiten

Buß I, Sarin N, Koegel S, Kassack M, Jaehde U, Kalayda GV. Role of organic cation transporters (hOCT1-3) and copper transporter (hCTR1) in influx of and resistance to oxaliplatin. (in Vorbereitung)

Buß I, Kalayda GV, Lindauer A, Reithofer MR, Galanski M, Keppler BK, Jaehde U. Reactivity affects cellular accumulation and cytotoxicity of platinum complexes. (in Vorbereitung)

Buß I, Garmann D, Galanski M, Weber G, Kalayda GV, Keppler BK, Jaehde U. Enhancing lipophilicity to overcome resistance against platinum complexes? *J Inorg Biochem*, submitted

Rotte A, Garmann D, Buß I, Jaehde U. Effect of Extracellular ATP on Cisplatin-Induced Cytotoxicity in Human Ovarian Carcinoma Cells. *Chemotherapy*. 2010, Epub ahead of print

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Buß I, Kalayda GV, Marques-Gallego P, Reedijk J, Jaehde U. Influence of the hOCT2 inhibitor cimetidine on the cellular accumulation and cytotoxicity of oxaliplatin. *Int J Clin Pharmacol Ther*. 2009, *47*, 51-4

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Übersichtsarbeiten und Buchbeiträge

Buß I. Arzneistoffprofile: Glibenclamid, Glimepirid, Metformin, Miglitol, Pioglitatzon, Repaglinid, Rosiglitazon. In: Ammon, Mutschler, Scholz: *Arzneimittelinformation und Beratung in der Apotheke*, Deutscher Apotheker Verlag, Stuttgart, in press

Buß I, Jaehde U. Platinum Complexes. In: Schwab M (Ed.): *Encyclopedia of Cancer*, Springer-Verlag, Berlin/Heidelberg, 2009

Abstracta/ Kongressmitteilungen

Buß I, Kalayda GV, Sarin N, Marques M, Jaehde U. (2008) Influence of Oxaliplatin on the Localization of Organic Cation Transporters (OCT). Jahrestagung der DPhG, Bonn (Poster)

Buß I, Kalayda GV, Jaehde U. (2008) Influence of Organic Cation Transporters (OCT) on the Uptake of Oxaliplatin. Jahrestagung der Central European Society of Anticancer Drug Research (CESAR), Göttingen (Kurzvortrag)

Buß I, Garmann D, Galanski M, Keppler BK, Jaehde U. (2007) Structure-Transport and Structure-Activity Relationships of Oxaliplatin Analogues. Jahrestagung der Central European Society of Anticancer Drug Research (CESAR), Freiburg (Kurzvortrag)

Buß I, Garmann D, Galanski M, Keppler BK, Jaehde U. (2007) Structure-Transport and Structure-Activity Relationships of Oxaliplatin Analogues. 13th International Conference on Biological Inorganic Chemistry, Wien (Poster)

Buß I, Garmann D, Galanski M, Keppler BK, Jaehde U. (2007) Structure-Activity Relationships of Oxaliplatin Analogues. Jahrestagung der DPhG, Erlangen, und Joint Meeting of Research Training Groups, Nürnberg (Poster)

Buß I, Reithofer MR, Galanski M, Keppler BK, Jaehde U. (2007) Structure-Transport and Structure-Activity Relationships of Oxaliplatin Analogues with Various Leaving Groups. 10th International Symposium on Platinum Coordination Compounds in Cancer Chemotherapy, Verona (Poster)

Buß I, Garmann D, Galanski M, Keppler BK, Jaehde U. (2006) Effect of lipophilicity on cellular uptake of platinum complexes. Doktorandentagung der Deutschen Pharmazeutischen Gesellschaft, 06. - 08. September 2006, Nürnberg (Poster)

Auslandsaufenthalte

15.07. - 20.07.2007	13th International Conference on Biological Inorganic Chemistry, Wien, Österreich (Poster)
30.11. - 03.12.2007	10th International Symposium on Platinum Coordination Compounds in Cancer Chemotherapy, Verona, Italien (Poster)

Promotionsbegleitende Tätigkeiten

Sommersemester 2009	Teilnahme an der Ringvorlesung „Klinische Prüfung von Arzneimitteln“ des Institutes für Klinische Chemie und Pharmakologie und des Bundesinstitutes für Arzneimittel und Medizinprodukte
Sommersemester 2009	Teilnahme an der Ringvorlesung „Arzneimittelentwicklung“ des Graduiertenkollegs
04/2009	Vortrag über die eigenen Befunde im Rahmen eines Minisymposiums des Graduiertenkollegs mit auswärtigen Gastwissenschaftlern
11/2008	Vortrag über die eigenen Befunde im zentralen Kolloquium des Graduiertenkollegs
06/2008	Teilnahme am Workshop „Fluoreszenzmikroskopie – Eine Methode zur Untersuchung zellulärer Systeme“
12/2007 und 06/2008	Leitung des Seminars „Zellkultur: Troubleshooting – Ärger mit den Zellen“ im Rahmen des Graduiertenkollegs
12/2007	Vortrag über die eigenen Befunde im zentralen Kolloquium des Graduiertenkollegs
09/2007	Teilnahme am BD Falcon™ Zellkulturkurs „Troubleshooting – Ärger mit den Zellen“, Homburg/ Saar
03/2007	Teilnahme am Workshop „Einführung in die pharmakokinetische und pharmakodynamische Datenanalyse“
01/2007	Teilnahme am Workshop „Biosynthese pharmakologisch aktiver Naturstoffe“
12/2006	Vortrag über die eigenen Befunde im zentralen Kolloquium des Graduiertenkollegs
11/2006	Vortrag zum Thema „Pharmazeutische Betreuung von Krebspatienten” im Rahmen der Wochenendworkshops der ABDA, Bremen
11/2006	Teilnahme am European Symposium on Patient Compliance and Persistance (ESPACOMP), Bonn

09/2006	Vortrag zum Thema „Gemeinsame Lehrveranstaltungen von Klinischer Pharmazie und Pharmakologie“ im Rahmen des Workshops Klinische Pharmazie der DPhG, Kiel
09/2006	Präsentation der eigenen Befunde im Rahmen des Internationalen Symposiums des Graduiertenkollegs (Posterpräsentation)
Sommersemester 2006	Teilnahme an der Ringvorlesung „Arzneimittelentwicklung“ des Graduiertenkollegs
03/2006	Projektvorstellung im Rahmen eines Minisymposiums des Graduiertenkollegs mit auswärtigen Gastwissenschaftlern (Posterpräsentation)
03/2006	Teilnahme am 27. Deutschen Krebskongress, Berlin
01/2006 – 08/2009	Tutorentätigkeit im Kursus Klinische Pharmazie